行政院國家科學委員會專題研究計畫　成果報告

數種中草藥對人類乳癌細胞增生抑制作用和細胞程式死亡機制之探討

計畫類別：個別型計畫
計畫編號：NSC93-2320-B-041-011-
執行期間：93 年 11 月 01 日至 94 年 07 月 31 日
執行單位：嘉南藥理科技大學生物科技系(所)

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一、中文摘要

本計劃篩選多個天然物之抗乳癌活性，結果以野玫瑰樹鹼(ellipticine)活性較佳，因此接續作深入機轉探討。結果發現野玫瑰樹鹼可以有效地藉由細胞週期 G2/M 的停滯和細胞凋亡的作用抑制乳癌細胞株 MDA-MB-231 細胞的增生。在細胞週期停滯方面，野玫瑰樹鹼可以降低 cyclinB1、Cdc2、Cdc25C 的表現，同時增加 Cdc2 的磷酸化因此使細胞週期停滯於 G2/M。在細胞凋亡的誘導方面，野玫瑰樹鹼可以增加 Bax 的表現，降低 Bcl-2 和 Bcl-XL 以及 XIAP 的表現而啓動粒線體相關的細胞凋亡途徑，包括使 cytochrome *c* 由粒線體釋放至細胞質、caspase-9 和 caspase-3 的活化。再者，利用 caspase-9 抑制劑將 caspase-9 活性阻斷有效的抑制野玫瑰樹鹼的細胞增生抑制和細胞凋亡誘導作用。因此確定粒線體途徑扮演重要的角色。本研究證明野玫瑰樹鹼爲具有防治乳癌潛力的天然物活性成分。

Abstract

Ellipticine, a cytotoxic plant alkaloid, is known to inhibit topoisomerase II. Here we report the mechanism of apoptosis induction and cell cycle arrest by ellipticine in human breast MDA-MB-231 cancer cells. Ellipticine treatment arrested MDA-MB-231 cells at the G2/M phase after 6 h of treatment. This effect was strongly associated with a concomitant decrease in the level of cyclin B1, Cdc25 and Cdc2, and increase in phospho-Cdc2 (Tyr15). In addition, ellipticine also induced apoptosis in MDA-MB-231 cells, as determined by using both DNA fragmentation and Annexin-V staining assay. Ellipticine increased the expression of Bax, but decreased the level of Bcl-2, Bcl-XL and X-linked inhibitor of apoptosis protein (XIAP), and subsequently triggered the mitochondrial apoptotic pathway (release of cytochrome *c*, and activation of caspase-9 and -3). In addition, pre-treatment of cells with caspase-9 inhibitor inhibited ellipticine-induced cell proliferation and apoptosis, indicating that caspase-9 activation was involved in MDA-MB-231 cell apoptosis induced by ellipticine. Taken together, our study suggests that the inhibition of cell cycle progression signaling

Introduction

Ellipticine (5,11-dimethyl-6H-pyrido[4,3-b]carbazole) is one of the simplest naturally occurring alkaloids, having a planar structure [1]. It was first isolated in 1959 from the leaves of the evergreen tree Ochrosia elliptica Labill (Apocynaceae), which grows wild in Oceania [1]. The anti-cancer activity of ellipticine and its derivatives, such as 9-methoxyellipticine, retelliptine and ellipticiniums, has been reported as being selectively active against cancer cells in both in vitro and in vivo studies [2–7]. Studies on the cytotoxicity and anti-cancer activity mechanisms of ellipticine and its analogs have shown these activities to be due to (i) DNA intercalation, (ii) inhibition of topoisomerase II, (iii) covalent alkylation of macromolecules and (iv) induction of endoplasmic reticulum stress [2–9]. The overall goal of our studies is to characterize the signaling pathways producing ellipticine-mediated cell growth inhibition, cell cycle arrest and apoptosis. The members of the ellipticine family may yield additional clinically useful anti-cancer drugs if their mechanisms of action and relative activities in tumors with particular molecular characteristics are better understood.

Materials and methods

Reagents and materials

Fetal calf serum (FCS), penicillin G, streptomycin, amphotericin B and Dulbecco's modified Eagle's medium (DMEM) were obtained from Gibco/BRL (Gaithersburg, Maryland, USA). Ellipticine, dimethylsulfoxide (DMSO), RNase and propidium iodide (PI) were purchased from Sigma (St Louis, Missouri, USA). XTT (sodium 30-[1-(phenylamino-carbonyl)-3,4-tetrazolium]-bis(4-methoxy-6-nitro) benzene-sulfonic acid hydrate was obtained from Roche Diagnostics (Mannheim, Germany). WAF1 ELISA, and caspase-9 and -3 activity assay kits were purchased from Calbiochem (Cambridge, Massachusetts, USA).The antibodies to cyclin B1, Cdc25, Cdc2, phospho-Cdc2 (Tyr15), Bcl-2, Bcl-XL, Bax, KIP1/p27 and X-linked inhibitor of apoptosis protein (XIAP) were obtained from Santa Cruz Biotechnology (Santa Cruz, California, USA).

Cell culture

Breast cancer cell line MDA-MD-231 was obtained from ATCC (Manassas, Virginia, USA). It was maintained in monolayer culture at 37℃ and 5% CO2 in DMEM supplemented with 10% FCS, 100 U/ml penicillin G, 100 mg/ml streptomycin and 0.25 mg/ml amphotericin B. All studies were performed with exponentially growing cells. MDA-MB-231 cells were seeded in cell culture plates or dishes. After 24 h, cells were treated with various concentration of ellipticine and incubated for the indicated times. Cells were harvested by trypsinization for flow cytometry analysis and scraped for other experiments.

Cell proliferation assay

Inhibition of cell proliferation by ellipticine was measured by the XTT assay. Briefly, cells were plated in 96-well culture plates (1×10^4 cells/well). After 24 h incubation, the cells were treated with vehicle control (0.1% DMSO) and ellipticine (0.5, 1, 2 and 3μM) for 48 h. Then 50 ml XTT test solution, which was prepared by mixing 5ml XTT labeling reagent with 100 ml electron-coupling reagent, was added to each well. After 4 h incubation, the absorbance was measured on an ELISA reader (Multiskan EX; Labsystems; Thermo Electron, Milford, Massachusetts, USA) at a test wavelength of 492 nm and a reference wavelength of 690 nm.

Cell cycle analysis

To determine cell cycle distribution analysis, 5×10^5 cells were plated in a 60-mm dish for 24 h and then treated with various doses of ellipticine (0, 1.5 and 3 μM) for 6 h. After treatment, the cells were collected by trypsinization, fixed in 70% ethanol, washed in phosphate-buffered saline (PBS), resuspended in 1ml of PBS containing 1 mg/ml RNase and 50 mg/ml propidium iodide, incubated in the dark for 30 min at room temperature, and analyzed by a flow cytometer (Epics Elite ESP; Coulter, Miami, FL). The data were analyzed using Multicycle software (Phoenix Flow Systems, San Diego, California, USA).

Apoptosis assay

Cells (1×10^6) were treated with vehicle alone (0.1%. DMSO) and various concentrations of ellipticine for 48 h, and collected by centrifugation. Pellets were lysed by DNA lysis buffer (10mM Tris, pH 7.5, 400mM EDTA and 1% Triton X-100) and then centrifuged. The supernatant obtained was incubated overnight with proteinase K (0.1 mg/ml) and then with RNase (0.2 mg/ml) for 2 h at 37℃. After extraction with phenol:chloroform (1:1), the DNA was separated in a 2% agarose gel and visualized by UV after staining with EB.

Quantitative assessment of apoptosis was also assessed by the BD ApoAlert Annexin V apoptosis kit (BD Biosciences, Boston, Massachusetts, USA) and analyzed by flow cytometry according to the manufacturer's instructions.

Assaying the levels of CIP/p21

The WAF1 ELISA was used for the detection of CIP/p21 levels. Briefly, cells were treated with vehicle alone (0.1% DMSO) or ellipticine (1.5 and 3 μM) for the indicated times. Samples of cell lysate were placed in 96-well (1×10^6/well) microtiter plates that were coated with monoclonal detective antibodies and incubated for 1 h at room at room

temperature. Upon removing unbound material by washing buffer, HRP-conjugated streptavidin was add to bind to the antibodies. HRP catalyzed the conversion of a chromogenic substrate to a colored solution with a color intensity proportional to the amount of protein present in the sample. The absorbance of each well was measured at 450nm and the level of CIP/p21 was determined by interpolating from standard curves obtained with known concentrations of standard proteins.

Assay for caspase activity

The assay is based on the ability of the active enzyme to cleave the chromophore from the enzyme substrates LEHD-pNA (for caspase-9) and Ac-DEVD-pNA (for caspase-3). The cell lysates were incubated with peptide substrate in assay buffer (100mM NaCl, 50mM HEPES, 10mM dithiothreitol, 1mM EDTA, 10% glycerol, 0.1% CHAPS, pH 7.4) for 2 h at 37℃. The release of p-nitroaniline was monitored at 405 nm. Results are represented as the percent change of the activity compared to the untreated control.

Western blotting assay

Cells (8×10^6/dish) were seeded in a 10-cm dish. After 24 h of incubation, the cells were treated with 3 mM ellipticine for the indicated times. Mitochondrial and cytoplasmic fractions were separated using the cytochrome *c* releasing apoptosis assay kit (BioVision, Mountain View, CA). Total cell extracts were prepared in lysis buffer (50mM Tris–HCl, 150 mM NaCl, 1mM EGTA, 1mM EDTA, 20mM NaF, 100 mM Na3VO4, 0.5% NP-40, 1% Triton X-100, 1mM PMSF, 5 mg/ml aprotinin and 5 mg/ml leupetin). Equivalent amounts of protein were resolved by SDS–PAGE (10–12%) and transferred to PVDF membranes. After the membrane was blocked in Tris-buffer saline containing 0.05% Tween 20 (TBST) and 5% non-fat powdered milk, the membranes were incubated with primary antibodies specific to cyclin B1, Cdc2, Cdc25, phosphor-Cdc2, Bax, Bcl-2, Bcl-XL, XIAP and cytochrome *c* at 41C for 1–16 h. After washing 3 times with TBST for 10 min each, the membrane was incubated with horseradish peroxidase-labeled secondary antibody for 1 h. The membranes were washed again and detection was performed using the enhanced chemiluminescence blotting detection system (Amersham, Piscataway, NJ).

Statistical analysis

Data were expressed as means±SD of three determinations. Statistical comparisons of the results were made using ANOVA. Significant differences ($p<0.05$) between the means of two test groups were analyzed by Dunnett's test.

Results

Effect of ellipticine on MDA-MB-231 cell proliferation inhibition.

We first determined the effect of ellipticine on the growth of MDA-MB-231 cells cultured for 48 h, either in the presence or absence of ellipticine, using the XTT assay. As shown in Figure 1, the growth of these MDAMB-231 cell lines was inhibited by ellipticine in a dose-dependent manner. The IC50 value was 1.82 μM.

Ellipticine-induced cell cycle arrest and apoptosis

in MDA-MB-231 cells. To examine the mechanism responsible for ellipticine mediated cell growth inhibition, the cell cycle distribution was evaluated using flow cytometric analysis. The result showed that the addition of 1.5μM ellipticine caused an accumulation of MDA-MB-231 cells in the G2/M phase at 6 h (28.12%) and this effect was significantly higher than with cells treated with 3 μM ellipticine (38.6%) (Fig. 2).

We next assessed the effect of ellipticine on the induction of apoptosis in MDA-MB-231 cells by the DNA fragmentation assay. The results showed that ellipticine treatment resulted in the formation of DNA fragments in MDA-MB-231 cells as assessed by agarose gel electrophoresis at 48 h (Fig. 3A). Additionally, a quantitative evaluation was then sought using an Annexin-V–FITC staining analysis which showed that treatment of MDA-MB-231 cells with ellipticine increased the percentage of apoptotic cells in a time dependent manner (Fig. 3B).

Ellitpticine alters the expression of cell cycle regulatory molecules

Since ellipticine inhibited cell cycle progression at the

G2/M phase, we assessed the expression levels of various cell cycle regulatory molecules, including CIP/p21, KIP1/p27, cyclin B1, Cdc25 and Cdc2. As shown in Figure 4(A), ellipticine failed to affect the expression of CIP/p21 at any examined time points. However, it increased the expression of KIP1/p27 at 3 h post-ellipticine addition (Fig. 4B). Ellipticine treatment of the cells resulted in a time-dependent decrease in the protein expression of cyclin B1 and Cdc25 as well as Cdc2. In addition, exposure to ellipticine for 3 h resulted in an increase in the level of phospho-Cdc2 (Tyr15). Results from time-dependent studies indicated that decreased Cdc25 expression by ellipticine was followed by an increase in phospho-Cdc2. We suggest that the decreased Cdc25 expression may be involved in the increase of inactivated form of phospho-Cdc2 (Tyr15) in ellipticine-treated MDA-MB-231 cells.

Involvement of the mitochondrial apoptotic pathway in ellipticine-mediated apoptosis

To investigate the mitochondrial apoptotic events involved in ellipticine-induced apoptosis, we first analyzed the changes in the levels of pro-apoptotic protein Bax, and anti-apoptotic proteins Bcl-2 and Bcl-XL levels. Western blotting analysis showed that treatment of MDA-MB-231 cells with ellipticine markedly increased Bax protein levels at 6 h (Fig. 5A). In contrast, ellipticine markedly decreased Bcl-2 levels at 6 h, which led to an increase in the Bax:Bcl-2 ratio (Fig. 5A). In addition, ellipticine also decreased the expression of Bcl-XL and XIAP at 3 h post-ellipticine addition (Fig. 5A). These effects of ellipticine on Bcl-2 family proteins led to decreased mitochondrial cytochrome *c* content and increased cytochrome *c* release into the cytosol (Fig. 5B).

Next, we investigated the implication of initiator caspases and effector caspases in ellipticine-induced apoptosis. Biochemical analysis showed that treatment with ellipticine increased caspase-9 activity in MDA-MB-231 cells, consistent with the release of cytochrome *c* into the cytosol (Fig. 5C). Furthermore, ellipticine subsequently increased effector caspase-3 activity (Fig. 5D).

The mitochondrial apoptotic pathway is required in ellipticine-mediated cell death

Next, we assessed the role of the mitochondrial apoptotic pathway in ellipticine-mediated apoptosis in MDA-MB- 231 cells. When cells were pre-treated with a caspase-9-specific inhibitor, LEHD-CHO (20 mM), for 1 h the antiproliferative and pro-apoptotic effects of ellipticine were effectively inhibited. At 3μM ellipticine, cell growth inhibition decreased from 87.53 to 21.4% (Fig. 6A). Compared to the control, the amount of apoptotic cells induced by 3μM ellipticine decreased from 73.4 to 19.8% at 48 h in MDA-MD-231 cells pre-treated with caspase-9 inhibitor (Fig. 6B).

Discussion

Ellipticine is a potent anti-neoplastic agent whose mechanism of action is considered to be based mainly on DNA intercalation and/or inhibition of topoisomerase II [2–9]. However, the molecular mechanism of its apoptotic effect as an anti-cancer agent has not yet been clarified. Our results demonstrate that ellipticine inhibits the growth of human breast MDA-MB-231 cancer cells. Treatment of MDA-MB-231 cells with ellipticine caused. The cells to accumulate in the G2/M phase of cell cycle and undergo apoptosis.

Eukaryotic cell cycle progression involves sequential activation of Cdks, whose activation is dependent upon their association with cyclins [10]. A complex formed by the association of Cdc2 (also known as cdk1 or $p34_{Cdc2}$) and cyclin B_1 plays a major role at the entry into mitosis [10]. The phosphorylation of Tyr15 of Cdc2 suppresses the activity of the Cdk1/cyclin B_1 kinase complex. Dephosphorylation of Tyr15 of Cdc2 is catalyzed by Cdc25 phosphatases and this reaction is believed to be the rate-limiting step for entry into mitosis [11]. Cell cycle progression is also regulated by the relative balance between the cellular concentration of cyclin-dependent kinase (CDK) inhibitors (CKIs), such as members of the CDK-interacting protein/CDK-inhibitory protein (CIP/KIP) and inhibitor of cyclin-dependent kinase (INK) families, and that of cyclin–CDK complexes. The Cip/Kip family, including CIP/p21 and KIP/p27, binds to cyclin–CDK complexes and prevents kinase activation, andsubsequently blocks

the progression of cell cycle at the G_0/G_1 or G_2/M phase [12,13]. In our result, we found that ellipticine treatment not only causes a significant reduction in the expression of cyclin B, Cdc25 and Cdc2 of MDA-MB-231 cells, but also decreases the activation of Cdc2 by preserving Tyr15 phospho-Cdc2. In addition, ellipticine treatment also increases the expression of KIP1/p27. Thus, it is reasonable to postulate that ellipticine treatment may cause cell cycle arrest by reducing the activity of the Cdc2–cyclin B kinase complex due to down-regulation of multiple G_2/M regulating proteins.

A number of pro- and anti-apoptotic members of the Bcl-2 protein family regulate the release of cytochrome *c* and apoptosis-inducing factor from the mitochondrial intermembrane space into the cytosol. Cytochrome *c* interacts with pro-caspase-9 and Apaf-1 to activate caspase-9, and then switches on caspase-3, -6 and -7, leading to apoptosis [14,15]. Ellipticine treatment was not only associated with an increase in the pro-apoptotic Bax protein level, but also a decrease in the anti-apoptotic Bcl-2 and Bcl-X_L amount, and subsequently also mediated the release of cytochrome *c*, finally activating the caspase cascade (caspase-9 and -3). Furthermore, the importance of this pathway was further confirmed by the partial protection of cell survival conferred by caspase-9 inhibition.

Taken together, our studies suggested that inhibition of cyclin B, Cdc25 and Cdc2, and its inactivation by increasing phosphorylation on Tyr15, was responsible for ellipticine-mediated cell cycle arrest. In addition, ellipticine (UA) not only alters the balance between proapoptotic Bax protein and anti-apoptotic Bcl-2, as well as Bcl-X_L, toward cell death, but also induces the release of cytochrome *c*, and activation of caspase-9 and -3, resulting in apoptosis.

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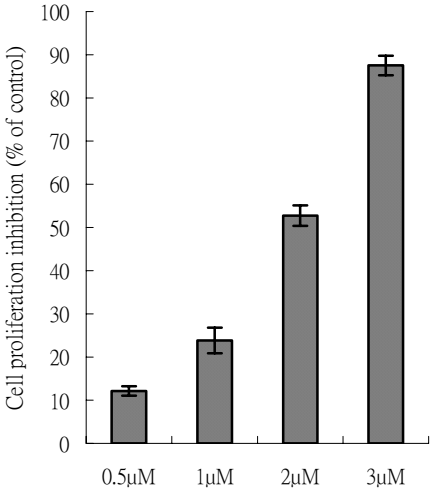


Figure 1. Effect of ellipticine on inhibiting the growth inhibition of MDA-MB-231 cells. Cells were seeded into 96-well plates (10^4cells/well) and allowed to adhere overnight. The next day, the cells were incubated with vehicle (0.1% DMSO) and different concentrations of ellipticine for 48 h. Cell proliferation was determined by XTT assay. Results are expressed as the percentage of cell proliferation relative to the proliferation of control. Each value is the mean ± S.D. of three determinations.

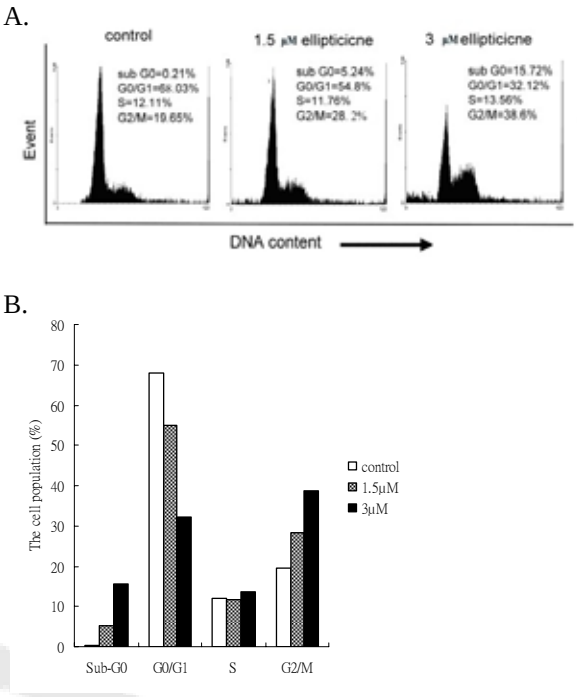
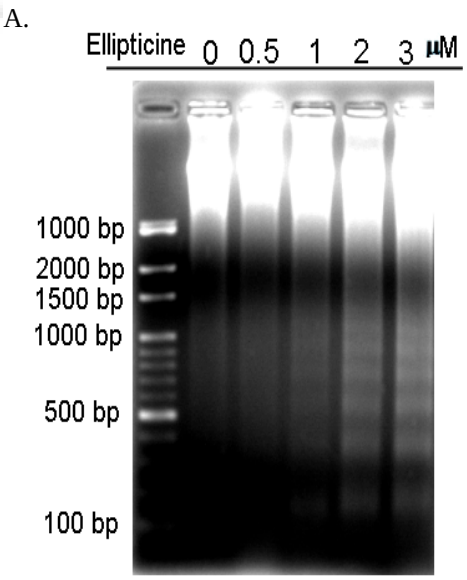


Figure 2. Effect of ellipticine on cell cycle distribution in MDA-MB-231 cells. (A) The flow cytometry histogram of MDA-MB-231 treated with or without ellipticine treatment. The distribution of cell population is expressed as percentage of cells counted and is shown in (B). Cells were treated with vehicle (0.1% DMSO), 1.5, and 3 μM ellipticine for 6 h, and the cell cycle distribution was assessed by flow cytometry.



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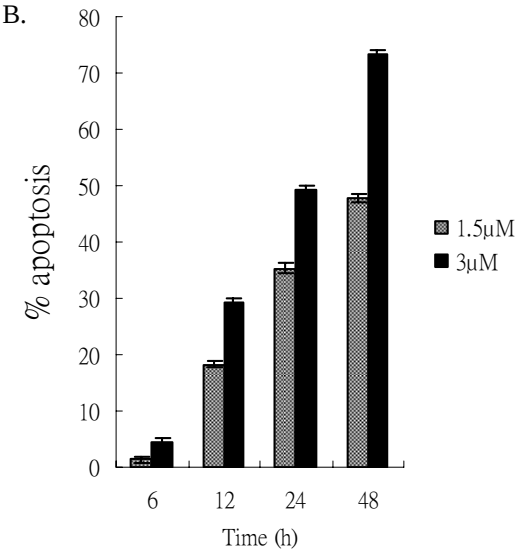
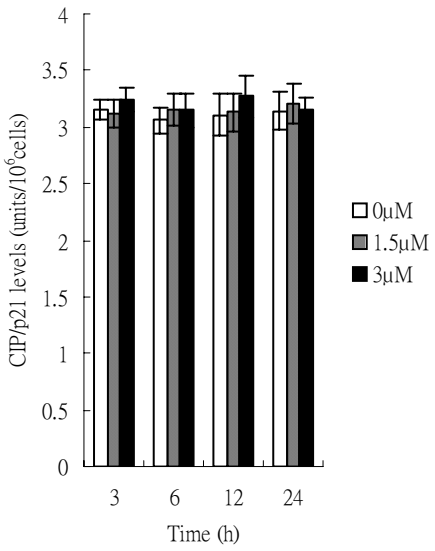


Figure 3. The effect of ellipticine on apoptosis induction in MDA-MB-231 cells. (A) The effect of ellipticine on DNA fragmentation in MDA-MB-231 cells. (B) The quantitation of apoptosis in ellipticine-treated cells. For (A), cells were treated with vehicle alone (0.1% DMSO) and various concentration of ellipticine for 48 h, and then the fragmentation of DNA was assessed by agarose gel electrophoresis. For (B), cells were treated 1.5, and 3 μM ellipticine for indicated times, and the cells were stained by Annexin V-FITC dye. Each value is the mean ± S.D. of three determinations. Results are expressed as the percentage of cell proliferation relative to the proliferation of control. Each value is the mean ± S.D. of three determinations.

A.



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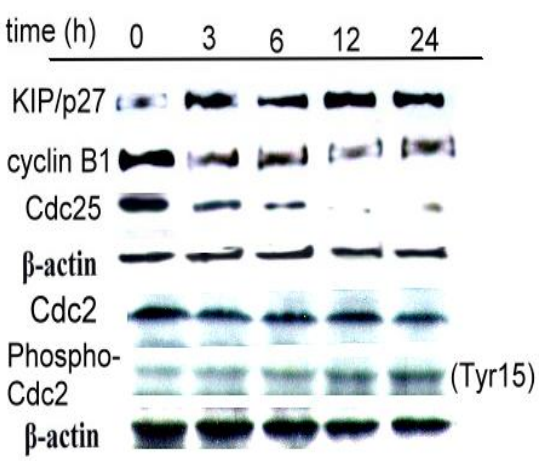
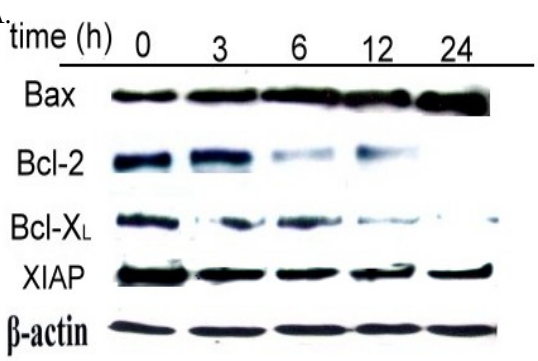


Figure 4. Effect of ellipticine on the expression of cell cycle-related molecules in ellipticine-treated MDA-MB-231 cells. (A) The level of CIP/p21. (B) The expression amount of KIP1/p27, cyclin B1, Cdc25 and Cdc2 as well as phospho-Cdc2 (Tyr15) in MDA-MB-231 cells. For (A), cells were treated with vehicle (0.1% DMSO), 1.5, and 3 μM ellipticine for the indicated times. The level of CIP/p21 protein was measured by WAF1 ELISA kit. Each value is the mean ± SD of three determinations. For (B), cells were treated with 3 μM ellipticine for indicated times. The expression amount of KIP1/p27, cyclin B1, Cdc25 and Cdc2 as well as phospho-Cdc2 (Tyr15) was determined by Western blotting.

A.



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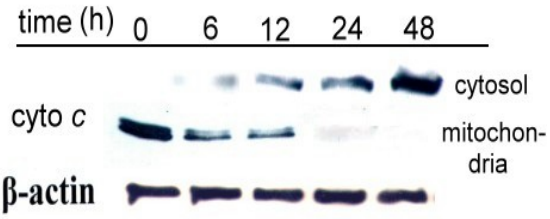

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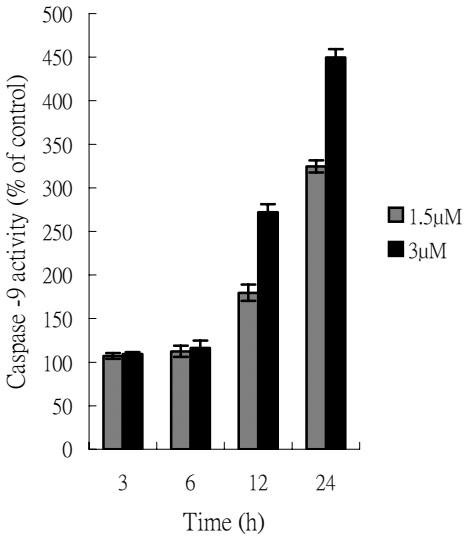

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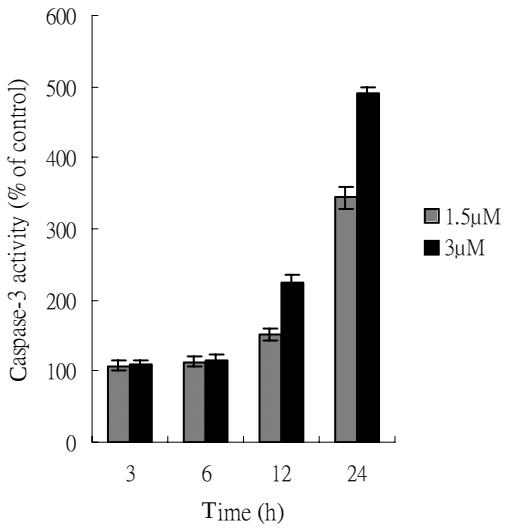

Figure 5. Ellipticine induced apoptosis through the initiation of the mitochondrial pathway. (A) The expression level of Bcl-2 family proteins and XIAP1/2 in ellipticine treated MDA-MB-231 cells. (B) The release of cytochrome from mitochondria into cytoplasm. (C) The activation of caspase-9 and (D) caspase-3 in ellipticine treated MDA-MB-231 cells. For (A) and (B), cells were treated with 3 μM ellipticine for indicated times. The extraction of cytoplasm and mitochondrial were separated from cell pellet by lysis buffer and centrifugation. Western blotting analysis assessed the protein expressions. For (C) and (D), the activity of caspase-9 and caspase-3 was assessed by caspase-9 and caspase-3 activity assay kit. Each value is the mean ± S.D. of three determinations.

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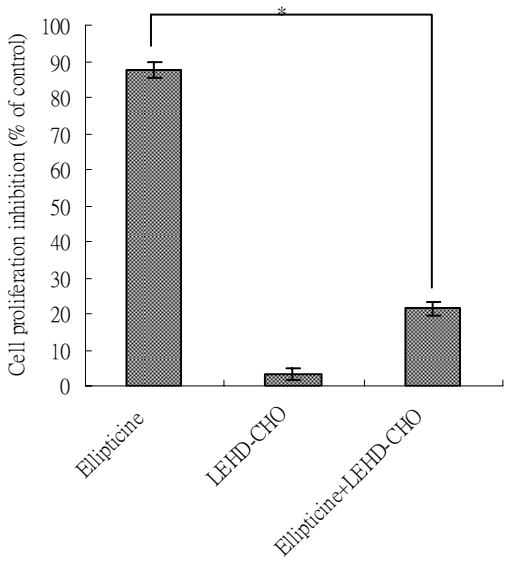

B.

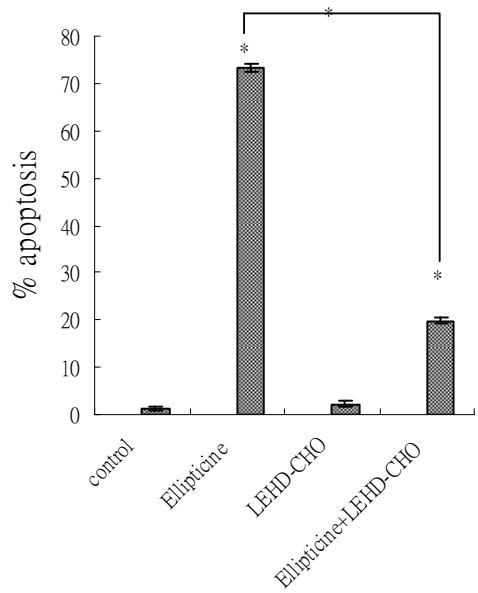

Figure 6. Specific inhibition of caspase-9 abrogated ellipticine-mediated (A) cell growth inhibition and (B) apoptosis in MDA-MB-231 cells. Cells were pre-treated with LEHD-CHO (20 μM) for 1 h before the addition of 3 μM ellipticine. After 48 h treatment, the cell viability and induction of apoptosis was measured by XTT and Annexin V staining kit. Each value is the mean ± SD of three determinations. The asterisk indicates a significant difference between two test group, * $p<0.05$.