The anti-proliferative inhibition of ellipticine in human breast mda-mb-231 cancer cells is through cell cycle arrest and apoptosis induction

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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 G_2/M phase after 6 h of treatment. This effect was strongly associated with a concomitant decrease in the level of cyclin B_1, 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-X_L 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 and initiation of the mitochondrial apoptotic system may participate in the anti-proliferative activity of ellipticine in MDA-MB-231 cells. *Anti-Cancer Drugs* 16:789–795 © 2005 Lippincott Williams & Wilkins.

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Introduction

Ellipticine (5,11-dimethyl-6*H*-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.

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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 3′-[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 B_1, Cdc25, Cdc2, phospho-Cdc2 (Tyr15), Bcl-2, Bcl-X_L, 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°C and 5% CO_2 in DMEM supplemented with 10% FCS, 100 U/ml penicillin G,

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100 μg/ml streptomycin and 0.25 μg/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 3 μM) for 48 h. Then 50 μl XTT test solution, which was prepared by mixing 5 ml XTT labeling reagent with 100 μl electron-coupling reagent, was added to each well. After a 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 1 ml of PBS containing 1 mg/ml RNase and 50 μg/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 (10 mM Tris, pH 7.5, 400 mM 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°C. After extraction with phenol:chloroform (1:1), the DNA was separated in a 2% agarose gel and visualized by UV after staining with ethidium bromide.

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.

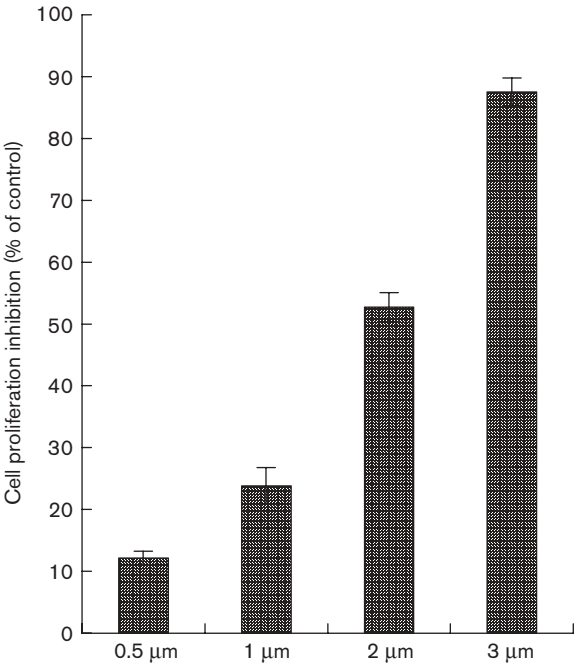
Assaying the levels of CIP/p21

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 temperature. Upon removing unbound material by washing with washing buffer, horseradish peroxidase-conjugated streptavidin was added to bind to the antibodies. Horseradish peroxidase catalyzed the conversion of a chromogenic substrate (tetramethylbenzidine) 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 450 nm 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 (100 mM NaCl, 50 mM HEPES, 10 mM dithiothreitol, 1 mM EDTA, 10% glycerol, 0.1% CHAPS, pH 7.4) for 2 h at 37°C. The release of

Fig. 1



Effect of ellipticine on inhibiting the growth inhibition of MDA-MB-231 cells. Cells were seeded into 96-well plates (10^4 cells/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 the XTT assay. Results are expressed as the percentage of cell proliferation relative to the proliferation of the control. Each value is the mean ± SD of three determinations.

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p-nitroaniline was monitored at 405 nm. Results are represented as the percent change of the activity compared to the untreated control.

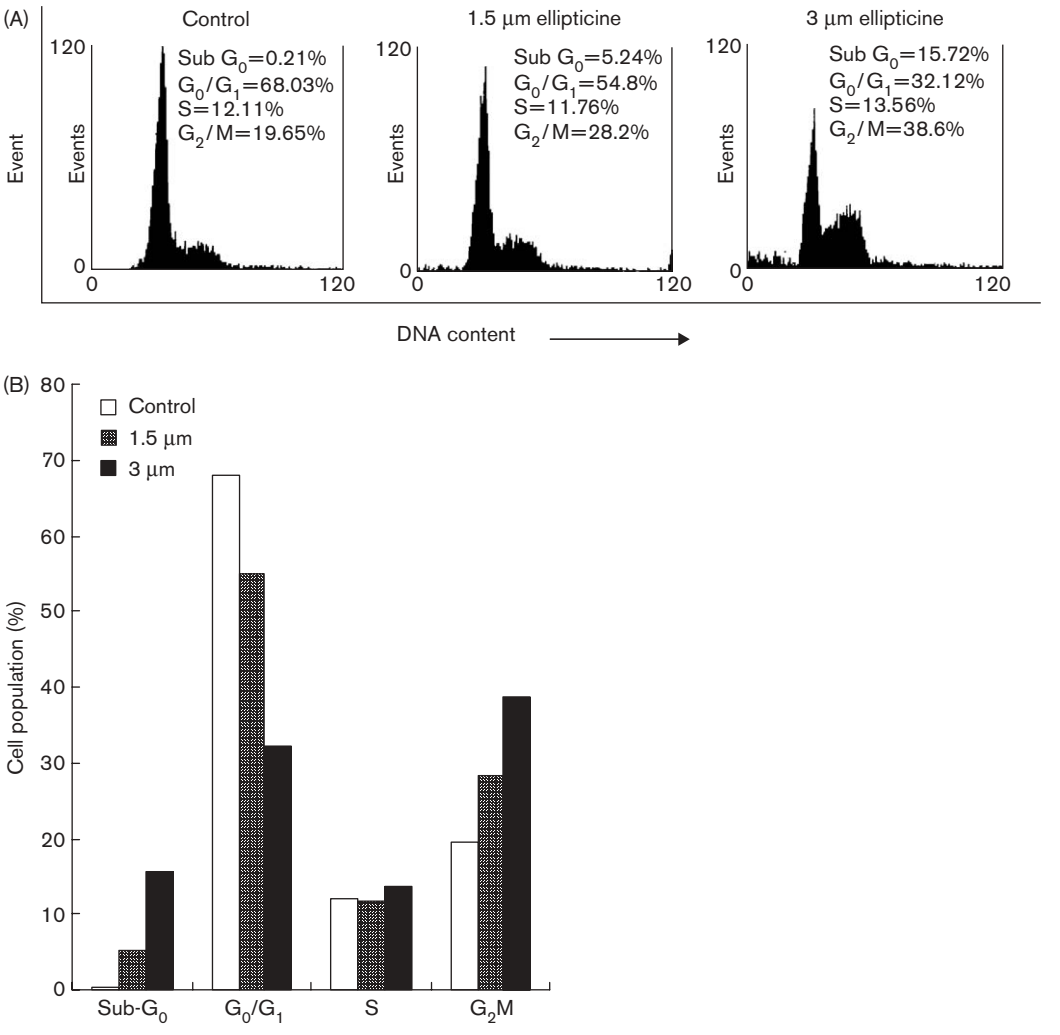
Western blotting assay

Cells (8×10^6/dish) were seeded in a 10-cm dish. After 24 h of incubation, the cells were treated with 3 μM 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 (50 mM Tris–HCl, 150 mM NaCl, 1 mM EGTA, 1 mM EDTA, 20 mM NaF, 100 mM Na_3VO_4, 0.5% NP-40, 1% Triton X-100, 1 mM PMSF, 5 μg/ml aprotinin and 5 μg/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 B_1, Cdc2, Cdc25, phosphor-Cdc2, Bax, Bcl-2, Bcl-X_L, XIAP and cytochrome *c* at 4°C 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.

Fig. 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 the cell population is expressed as percentage of cells counted and is shown in (B). Cells were treated with vehicle (0.1% DMSO), and 1.5 and 3 μM ellipticine for 6 h, and the cell cycle distribution was assessed by flow cytometry.

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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 MDA-MB-231 cell lines was inhibited by ellipticine in a dose-dependent manner. The IC_{50} 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 1.5 μM ellipticine caused an accumulation of MDA-MB-231 cells in the G_2/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-independent manner (Fig. 3B).

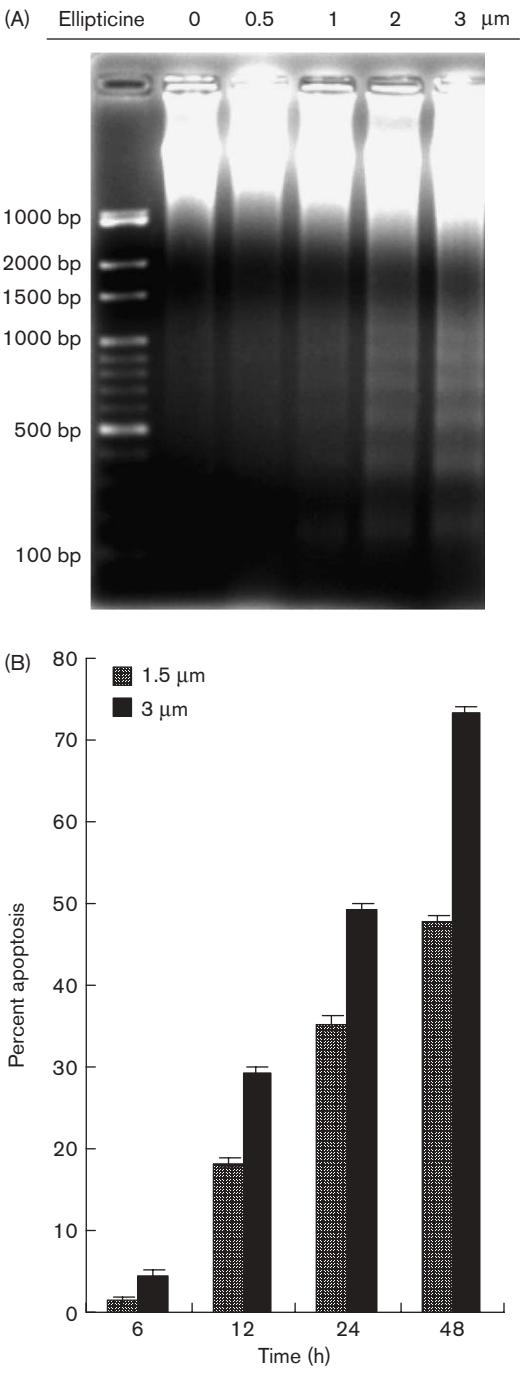
Ellitpticine alters the expression of cell cycle regulatory molecules

Since ellipticine inhibited cell cycle progression at the G_2/M phase, we assessed the expression levels of various cell cycle regulatory molecules, including CIP/p21, KIP1/p27, cyclin B_1, 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 B_1 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

Fig. 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 concentrations of ellipticine for 48 h, and then the fragmentation of DNA was assessed by agarose gel electrophoresis. For (B), cells were treated with 1.5 and 3 μM ellipticine for the indicated times, and the cells were stained by Annexin-V–FITC dye. Each value is the mean ± SD of three determinations. Results are expressed as the percentage of cell proliferation relative to the proliferation of the control. Each value is the mean ± SD of three determinations.

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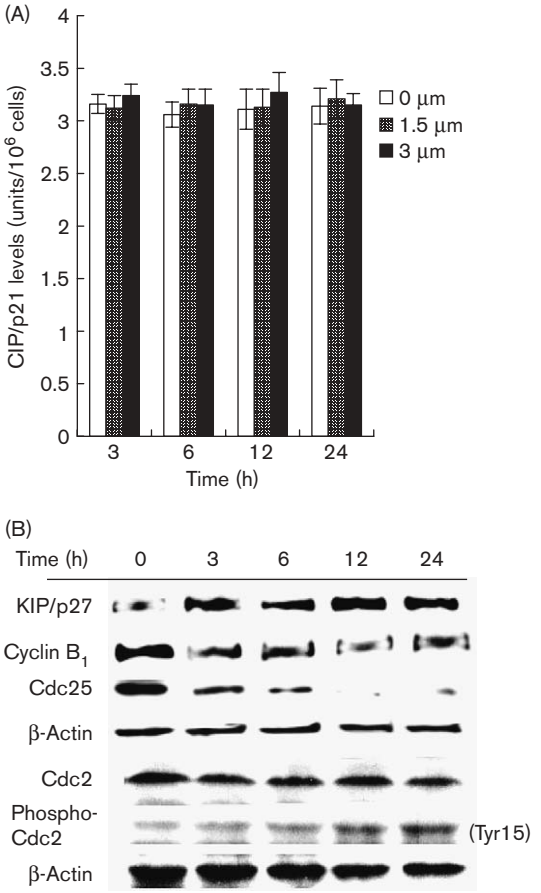
Fig. 4



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 amount of KIP1/p27, cyclin B_1, Cdc25 and Cdc2 as well as phospho-Cdc2 (Tyr15) expressed in MDA-MB-231 cells. For (A), cells were treated with vehicle (0.1% DMSO), and 1.5 and 3 μM ellipticine for the indicated times. The level of CIP/p21 protein was measured by the WAF1 ELISA kit. Each value is the mean ± SD of three determinations. For (B), cells were treated with 3 μM ellipticine for the indicated times. The expression of KIP1/p27, cyclin B_1, Cdc25 and Cdc2 as well as phospho-Cdc2 (Tyr15) was determined by Western blotting.

the changes in the levels of pro-apoptotic protein Bax, and anti-apoptotic proteins Bcl-2 and Bcl-X_L 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-X_L 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 μM), for 1 h the anti-proliferative and pro-apoptotic effects of ellipticine were effectively inhibited. At 3 μM ellipticine, cell growth inhibition decreased from 87.53 to 21.4% (Fig. 6A). Compared to the control, the amount of apoptotic cells induced by 3 μM 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 G_2/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, and subsequently 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

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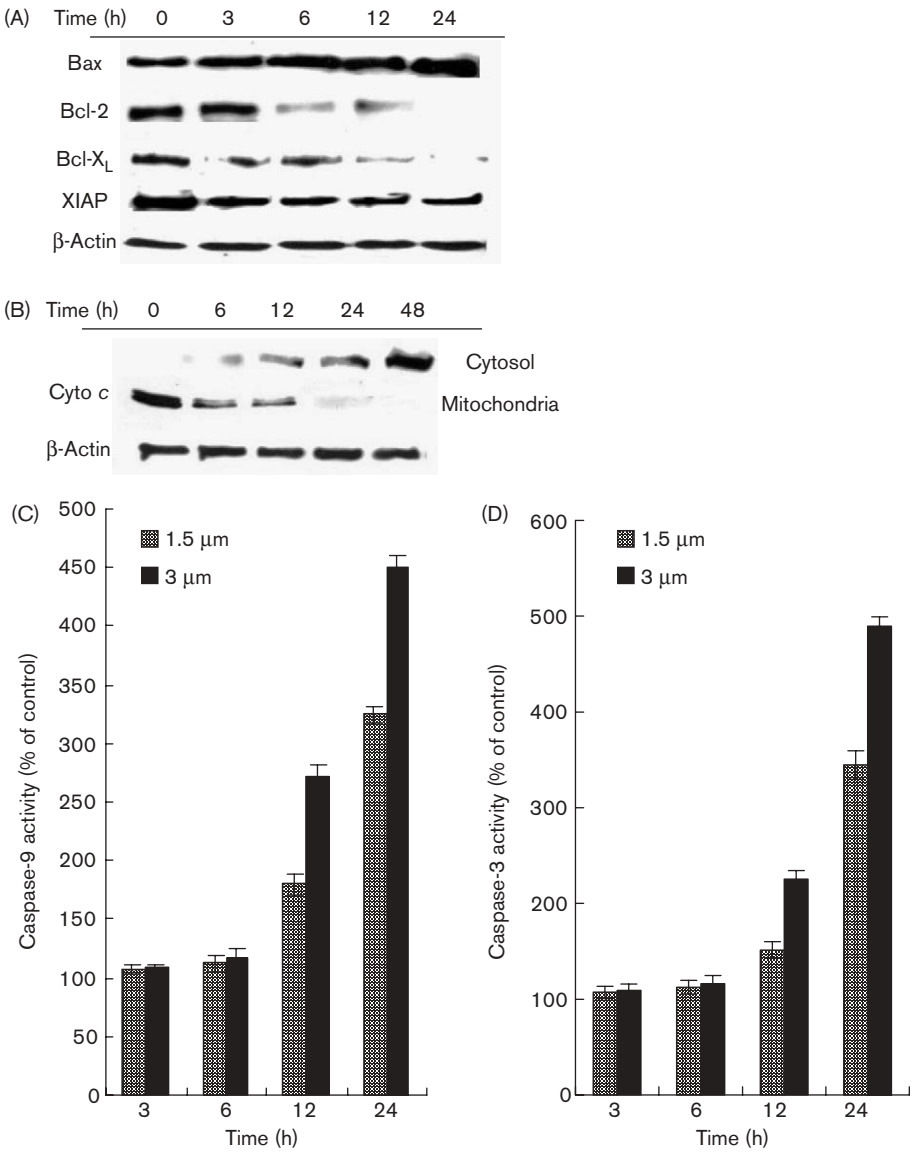
Fig. 5



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

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.

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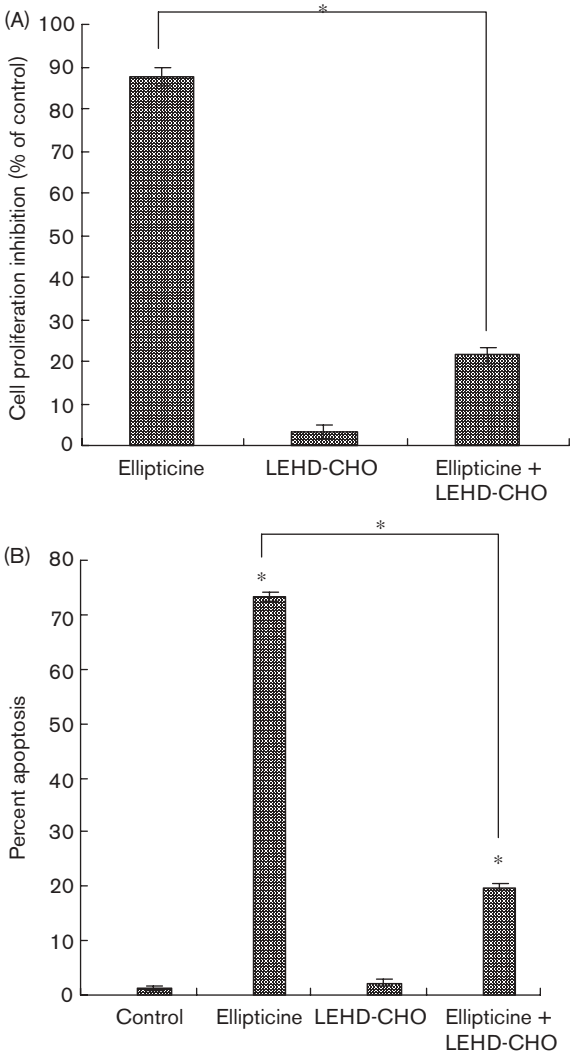
Fig. 6



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, cell viability and induction of apoptosis were measured by the XTT and Annexin-V staining kits. Each value is the mean ± SD of three determinations. The asterisk indicates a significant difference between two test group, $*p<0.05$.

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 pro-apoptotic 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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