The mechanism of ellipticine-induced apoptosis and cell cycle arrest in human breast MCF-7 cancer cells

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Abstract

Ellipticine, a cytotoxic plant alkaloid, is known to inhibit topoisomerase II. Here, we first report the molecular mechanism of ellipticine’s apoptotic action in human breast MCF-7 cancer cells. Treatment of cells with ellipticine resulted in inhibition of growth, and G2/M phase arrest of the cell cycle. This effect was associated with a marked increase in the protein expression of p53 and p21/WAF1 and KIP1/p27, but not of WAF1/p21. Ellipticine treatment increased the expression of Fas/APO-1 and its ligands, mFas ligand and sFas ligand, and subsequent activation of caspase-8. The mitochondrial apoptotic pathway amplified the Fas/Fas ligand death receptor pathway by Bid interaction. This effect was found to result in a significant increase in activation of caspase-9. Taken together, we have concluded that the molecular mechanisms during ellipticine-mediated growth inhibition and induction of apoptosis in MCF-7 cells were due to (1) cell cycle arrest and induction of apoptosis, (2) induction of p53 and KIP1/p27 expression, (3) triggering of Fas/Fas ligand pathway, (4) disruption of mitochondrial function, and (5) the apoptotic signaling was amplified by cross-talk between Fas death receptor and mitochondrial apoptotic pathway. © 2004 Elsevier Ireland Ltd. All rights reserved.

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1. 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 \textit{Ochrosia elliptica} Labill (Apocynaceae), which grows wild in Oceania [1]. The anticancer activity of ellipticine and its derivatives, such as 9-methoxyellipticine, retelliptine, ellipticiniums, have been reported as being selectively active against cancer cells in in vitro and in vivo studies [2–7]. Studies on the mechanisms of the cytotoxicity and anticancer activity of ellipticine and its analogs have shown that these activities to be due to (1) DNA intercalation, (2) inhibition of topoisomerase II, (3) covalent
alkylation of macromolecules, and (4) induction of endoplasmic reticulum stress [2–9].

Apoptosis signaling converges at the activation of initiator caspases (i.e. caspase-8 and caspase-9), which leads to the proteolytic activation of effectors caspase (i.e. caspase-3) that thencleaves the cellular substrate, resulting in cell death [10,11]. The death receptor pathway is triggered by members of the death receptor superfamily, such as Fas/APO-1. Ligation of Fas/APO-1 by agonistic antibody or its mature ligand (Fas ligand) induces receptor oligomerization and formation of death-inducing signaling complex (DISC), followed by activation of caspase-8. The mitochondrial apoptotic pathway is controlled by Bcl-2 family protein, including the proapoptotic Bax and antiapoptotic Bcl-2 and Bcl-X 

In this study, to establish the anticancer mechanism of ellipticine in MCF-7 cells, we assayed the death receptor and mitochondrial apoptotic pathway-related molecules, including Fas/APO-1, Fas ligand, caspase-8, cytochrome c, caspase-9, and Bcl-2 family proteins, which are strongly associated with the signal transduction pathway of apoptosis and affect the chemosensitivity of tumor cells to anticancer agents.

2. Materials and methods

2.1. Materials

Fetal calf serum (FCS), penicillin G, streptomycin, amphotericin B, Dulbecco’s modified Eagle’s medium (DMEM) and insulin were obtained from GIBCO BRL (Gaithersburg, MD). Ellipticine, dimethyl sulfoxide (DMSO), ribonuclease (RNase), and propidium iodide (PI) were purchased from Sigma Chemical Co. (St Louis, MO). XTT and p53 pan ELISA kits were obtained from Roche Diagnostics GmbH (Germany). Nucleosome ELISA, WAF1 ELISA, Fas Ligand, Fas/APO-1 ELISA, and caspase-8, caspase-9 activity assay kits, caspase-8 inhibitor (Z-IETD-FMK) and caspase-9 inhibitor (LEDH-CHO) were purchased from Calbiochem (Cambridge, MA). The antibodies to KIP/p27, Bax, Bcl-2, and Bcl-X 

2.2. Cell line and culture

MCF-7 (American Type Culture Collection [ATCC] HB8065) was maintained in a monolayer culture at 37 °C and 5% CO₂ in DMEM supplemented with 10% FCS, 10 U/mL of penicillin, 10 μg/mL of streptomycin, and 0.25 μg/mL of amphotericin B and 5 μg/mL of insulin.

2.3. Cell proliferation assay

Inhibition of cell proliferation by ellipticine was measured by XTT (sodium 3-[1-(phenylamino-carbonyl)-3,4-tetrazolium]-bis(4-methoxy-6-nitro)benzene-sulfonic acid hydrate) assay. Briefly, cells were plated in 96-well culture plates (1×10⁵ cells/well). After 24 h incubation, the cells were treated with ellipticine (0.5, 1, 2, and 3 μM) for 48 h. Fifty microliters of XTT test solution, which was prepared by mixing 5 mL of XTT-labeling reagent with 100 μL of electron coupling reagent, was then added to each well. After 4 h incubation, the absorbance was measured on an ELISA reader (Multiskan EX, Labsystems) at a test wavelength of 492 nm and a reference wavelength of 690 nm.

2.4. Cell cycle analysis

To determine cell cycle distribution analysis, 5×10⁵ cells were plated in 60-mm dishes and treated with ellipticine (0, 1.5, and 3 μM) for 6 h. After treatment, the cells were collected by trypsinization, fixed in 70% ethanol, washed in 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 EPICS flow cytometer. The data were analyzed using the Multicycle software (Phoenix Flow Systems, San Diego, CA).
2.5. Apoptosis assay

Cells (1×10⁶) were treated with vehicle alone (0.1% DMSO) and various concentrations of ellipticine for 48 h, and then 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 2% agarose gel and visualized by UV after staining with ethidium bromide.

Quantitative assessment of oligonucleosomal DNA fragmentation was assayed using the Nucleosome ELISA kit. Briefly, the cells were treated with vehicle alone (0.1% DMSO) and ellipticine (1.5 and 3 μM) for the indicated times. The induction of apoptosis was evaluated by assessing the enrichment of nucleosome in cytoplasm, and determined exactly as described in the manufacturer’s protocol.

2.6. Assaying the levels of p53, p21/WAF1, Fas ligand (mFasL and sFasL) and Fas/APO-1

p53 pan ELISA, WAF1 ELISA, Fas Ligand and Fas/APO-1 ELISA kits were used for the detection of p53, p21/WAF1, Fas ligand (mFasL and sFasL) and Fas/APO-1 receptor. Briefly, cells were treated with vehicle alone (0.1% DMSO) and ellipticine (1.5 and 3 μM) for the indicated times. Samples of cell lysate were placed in 96 well (1×10⁶ per well) microtiter plates that were coated with monoclonal detective antibodies, and incubated for 1 h (Fas/APO-1), 2 h (p53 or p21/WAF1) or 3 h (FasL) at room temperature. The soluble Fas ligand in cell culture supernatant also needed to be determined by using a Fas Ligand ELISA kit. Upon removing unbound material by washing with 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 concentrations of p53, p21/WAF1, FasL and Fas/APO-1 were determined by interpolating from standard curves obtained with known concentrations of standard proteins [15,16].

2.7. Assay for caspase activity

The assay is based on the ability of the active enzyme to cleave the chromophore from the enzyme substrate, Ac-IETD-pNA (for caspase-8), and LEHD-pNA (for caspase-9). 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 p-nitroaniline was monitored at 405 nm. Results are represented as the percent change of the activity compared to the untreated control.

2.8. Western blotting

Cells (8×10⁶/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 Cytochrome c Releasing Apoptosis Assay Kit (BioVision, California, USA). Total cell extracts were prepared in lysis buffer (50 mM Tris–HCl, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 20 mM NaF, 100 mM Na3VO4, 0.5% NP-40, 1% Triton X-100, 1 mM PMSF, 5 μg/mL Aprotinin, 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 (TBST) containing 0.05% Tween 20 and 5% nonfat powdered milk, the membranes were incubated with primary antibodies specific to Bax, Bid (full and cleaved length), Bcl-2, Bcl-XL, and cytochrome c at 4 °C for 16 h. After washing three 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, USA).

2.9. Statistical analysis

Data were expressed as means ± SD. Statistical comparisons of the results were made using analysis of variance (ANOVA). Significant differences
3. Results

3.1. Effect of ellipticine on MCF-7 cell growth inhibition

We first determined the effect of ellipticine on growth of MCF-7 cells cultured for 48 h, either in the presence or absence of ellipticine, using XTT assay. As shown in Fig. 1, the growth of these MCF-7 cell lines was inhibited by ellipticine in a dose-dependent manner. The IC_{50} value was 1.52 μM.

3.2. Ellipticine-induced cell cycle arrest and apoptosis in MCF-7 cells

To examine the mechanism responsible for ellipticine-mediated cell growth inhibition, cell cycle distribution was evaluated using flow cytometric analysis. Compared with the vehicle-treated control, ellipticine treatment resulted in an appreciable arrest of cells in G2/M phase of cell cycle after 6 h of the treatment. The treatment caused an arrest of 30.3% cells in the G2/M phase of the cell cycle at 1.5 μM concentration that further 39.1% at 3 μM in MCF-7 cells (Fig. 2).

We next assessed the effect of ellipticine on the induction of apoptosis in MCF-7 cells by DNA fragmentation assay. The results showed that ellipticine treatment results in the formation of DNA fragments in MCF-7 cells, as assessed by agarose gel electrophoresis at 48 h (Fig. 3A). Additionally, a quantitative evaluation was then sought using an ELISA to detect histone-associated oligonucleosome DNA fragments. Compared with vehicle-treated cells, 1.5 μM ellipticine induced 3.5 and 5.29-fold of oligonucleosome in MCF cells at 24 and 48 h. This effect was more pronounced with 3 μM ellipticine treatment (Fig. 3B).

3.3. Ellipticine increases the expression of p53 and KIP1/p27, but not WAF1/p21 proteins in MCF-7 cells

To determine whether the cell growth inhibition effects of ellipticine are mediated through changes in several key cell cycle regulatory factors, we examined the effects of treatment on the cell cycle regulatory proteins p53, WAF1/p21, and KIP1/p27 in MCF-7 cells (Fig. 4A–C). Treatment with ellipticine resulted in an increase of p53 and KIP1/p27 that was observed at 3 h and persisted until 24 h. However, treatment of MCF-7 cells with 1.5 or 3 μM ellipticine failed to show any significant effects on the levels of WAF1/p21 proteins at any examined times point. These data suggest that one potential mechanism of action of the observed anticancer effects of ellipticine...
may be through the up-regulation of p53 and the cell cycle regulatory proteins KIP1/p27, but not WAF/p21.

3.4. Fas/FasL apoptotic system might be a possible pathway of ellipticine-mediated apoptosis

Fig. 5A shows that treatment of MCF-7 cells with ellipticine increased Fas/APO-1 protein levels; maximum enhancement was observed within 12 h of exposure to the drugs. Compared to the control, 3 μM ellipticine increased 2.9, 4.2, and 3.1-fold of Fas/APO-1 at 6, 12, and 24 h, respectively. Results on Fas ligand assay indicate that FasL, mFasL, and
sFasL increased in a dose-dependent manner (Fig. 5B and C). The accumulation of mFasL was observed at 6 h after ellipticine treatment, and increased progressively for up to 12 h (Fig. 5B). A similar result was observed for sFasL (Fig. 5C). However, the amount of mFasL by ellipticine was more than sFasL at all time points.

We next measured the initiator caspase-8 of the Fas/FasL apoptotic system. The results showed that caspase-8 activity increased at 6 h, and reached maximum induction at 12 h in ellipticine treated MCF-7 cells (Fig. 5D). Furthermore, our results showed that the ellipticine’s cell growth inhibition and apoptosis induction decreased significantly in
the presence of caspase-8 inhibitor (Z-IETD-FMK) (Fig. 5E and F).

3.5. Mitochondrial apoptotic pathway is involved 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. Western blot analysis showed that treatment of MCF-7 cells with ellipticine increased Bax protein levels (Fig. 6A). In contrast, ellipticine markedly decreased Bcl-2 levels, which led to an increase in the Bax/Bcl-2 ratio (Fig. 5A). In addition, ellipticine also decreased the expression of Bcl-XL. These effects of ellipticine on Bcl-2 family proteins led to the release of mitochondrial cytochrome c content into the cytosol (Fig. 6A). Next, we investigated the implication of initiator caspases and effector caspases in ellipticine-induced apoptosis. Biochemical analyses showed that treatment with ellipticine increased caspase-9 activity in MCF-7 cells, consistent with the release of cytochrome c into the cytosol (Fig. 6B).

3.6. The activation of the mitochondrial pathway was through caspase-8-mediated Bid cleavage

Since ellipticine-mediated apoptosis involved initiation of Fas/Fas ligand death receptor and mitochondrial signaling, it is possible that ellipticine activates the mitochondrial apoptotic pathway through caspase-8-mediated Bid cleavage, which then results in cytochrome c release and caspase-9 activation. To test this idea, we checked the status of Bid protein during ellipticine-induced apoptosis. Fig. 5D shows that full size Bid (22 kDa) protein was cleaved to yield a 15 kDa fragment after treatment of cells with ellipticine, and this closely matched the appearance of caspase-8 activation. To assess whether proteolytic cleavage by caspase-8 is really responsible for the truncation of Bid, we investigated the effects of various caspase inhibitors on Bid cleavage. When cells were pre-treated with the specific caspase-8 inhibitor Z-IETD-fmk before ellipticine treatment, the decreased level of Bid protein recovered in ellipticine-treated cells was near that of the untreated control cells (Fig. 7). However, pre-treatment with the specific caspase-9 inhibitor LEHD-CHO did not prevent the disappearance of native Bid protein (Fig. 7). These results

![Fig. 6. Ellipticine-induced apoptosis through the initiation of the mitochondrial pathway. (A) The release of cytochrome c and the expression level of Bcl-2 family proteins in ellipticine treated MCF-7 cells. (B) The activation of caspase-9 in ellipticine treated MCF-7 cells. (C) The cleavage of Bid in ellipticine treated MCF-7 cells. For (A), cells were treated with 3 μM ellipticine for the indicated times. Cytoplasm and mitochondria were separated from the cell pellets by lysis buffer and centrifugation. Western blotting analysis assessed protein expressions. For (B), the activity of caspase-9 was assessed by a caspase-9 activity assay kit. Each value is the mean ± SD of three determinations. The cleavage of Bid was assessed by Western blotting analysis.](image)
indicate that Bid protein is cleaved by caspase-8, which is activated by ellipticine treatment, and that the activation of caspase-9 may be a downstream event of this caspase-8-mediated Bid cleavage.

4. 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 for its apoptotic effect as an anticancer agent has not yet been clarified. Our results demonstrate that ellipticine inhibits the growth of human breast MCF-7 cancer cells. Treatment of MCF-7 cells with ellipticine accumulated at the G2/M phase of cell cycle and underwent apoptosis. We found that ellipticine increased the expression of p53 and KIP1/p27, but did not affect the expression of WAF1/p21, suggesting that up-regulated of p53 and KIP1/p27 might contribute to ellipticine’s blockade effect on cell cycle progression at the G2/M phase.

Numerous studies have reported that the involvement of p53 in ellipticine-mediated cytotoxic effect [17–19]. Ellipticine has been found to restore the transcription function of mutant p53 (175 H, 248W, 249S, 273 H, 281G, 233L, 241F, and 273C mutants), and this property may contribute to resisting tumor cell lines expressing mutant p53 [17]. 9-Hydroxyellipticine (9-HE) treatment caused induction of apoptosis in the G1 phase of the cell cycle in mutant p53 (p53ala143, p53his175, orp53his273) transfected Saos-2 cells, but not in p53-deficient parental Saos-2 cells. Similar induction of apoptosis was observed 24–48 h after treatment with 9-HE in mutant p53-containing SW480, SK-BR-3 and MKN-1, but not in p53-deficient KATO cells [18]. Ellipticine and 9-HE caused selective inhibition of p53 protein phosphorylation in Lewis lung carcinoma and SW480 (human colon cancer cell line) [19]. In our study, we found that the expression of p53 increased in ellipticine treated MCF-7 cells. Two downstream molecules of p53, Fas/APO-1 and Bax, were accumulated after p53 increased by treatment with ellipticine. However, the expression of WAF1/p21 known to be regulated by p53 dose not increase in ellipticine-treated MCF-7 cells. Therefore, the actual role of p53 in ellipticine-mediated apoptosis in MCF-7 cells needs additional investigation in further studies.

In our ongoing efforts to determine the apoptotic mechanism of ellipticine against MCF-7 cells, we studied the involvement of Fas/Fas ligand death receptor and the mitochondrial pathway. Our study indicates that the Fas ligands mFasL and sFasL increase in ellipticine-treated MCF-7 cells. Fas/APO-1 levels and caspase-8 activity are simultaneously enhanced in FasL-upexpressing MCF-7 cells. Furthermore, cell growth inhibition and apoptotic induction of ellipticine decreases in MCF-7 cells treated with caspase-8 inhibitor. On the other hand, we found that ellipticine increased the expression of Bax and decreased the expression of Bcl-2 and Bcl-XL in MCF-7 cells, and subsequently induced the release of cytochrome c from the mitochondria into the cytoplasm, then activated caspase-9. Furthermore, we have also demonstrated that cross-talk between Fas/Fas ligand and the mitochondrial pathway is provided by Bid in ellipticine-treated MCF-7 cells. The cleavage of Bid by caspase-8 supports the hypothesis that the proteolysis event of Bid is suppressed by caspase-8 inhibitor pre-treatment, but not in caspase-9 inhibitor pre-treated MCF-7 cells.

In summary, 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 might be adopted clinically as useful anticancer drugs if their mechanisms of action and related activities in tumors with particular molecular characteristics are better understood. Our studies prove that induction of p53, Fas/Fas ligand death receptor, and mitochondrial...
proapoptotic pathways are involved in ellipticine-mediated cell growth inhibition in MCF-7 cells.

References


