Deltonin induces apoptosis in MDA-MB-231 human breast cancer cells via reactive oxygen species-mediated mitochondrial dysfunction and ERK/AKT signaling pathways

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Abstract. Deltonin, a steroidal saponin isolated from Dioscorea zingiberensis Wright, exhibits high cytotoxic activity in cancer cells. In the present study, the effects of deltonin on cell proliferation and apoptosis were evaluated in the MDA-MB-231 human breast carcinoma cell line. Following treatment with deltonin, the viability of MDA-MB-231 cells was analyzed using MTT assay and apoptosis, mitochondrial membrane potential (ΔΨm) alternation and intracellular reactive oxygen species (ROS) generation was determined by flow cytometry. In addition, western blot analysis was performed to examine the expression of apoptosis-associated proteins. The results demonstrated that deltonin induced apoptosis in MDA-MB-231 cells in a time- and concentration-dependent manner. Apoptosis was associated with depolarization of ΔΨm and time-dependent ROS generation. Deltonin treatment also resulted in Bax upregulation, Bcl-2 downregulation, activation of caspase-3 and -8 and poly (ADP ribose) polymerase cleavage. Decreased levels of phosphorylated extracellular signal-regulated kinase (ERK) and phosphorylated AKT were also observed. Results indicate that the proliferation inhibitory effect of deltonin is associated with its apoptosis-inducing effect, which may correlate with ROS-mediated mitochondrial dysfunction as well as activation of the ERK/AKT signaling pathways. Therefore, deltonin may be a potential chemotherapeutic agent for the treatment of breast cancer.

Introduction

Breast cancer is the most commonly diagnosed cancer and the leading cause of cancer mortality among females, accounting for 23% of total cancer cases and 14% of cancer mortalities (1). In developing countries, including China, breast cancer remains a significant public health issue due to its prevalence (2). In recent years, medical advances have increased the availability and efficacy of breast cancer treatments, including improved surgical methods for lumpectomy and mastectomy and radiation, hormone and chemotherapies. However, the majority of drugs currently used as chemotherapeutic agents exhibit low efficacy and are associated with the development of drug resistance (3,4). Therefore, new drugs with a higher therapeutic index are urgently required to effectively treat this malignancy.

Over the last two decades, an increasing number of bioactive compounds have been identified in traditional Chinese medical herbs (5). Specific compounds have been reported to kill tumor cells by generation of reactive oxygen species (ROS). ROS are known to affect mitochondrial membrane potential (ΔΨm) and trigger a series of mitochondria-associated events (6,7). The generation of ROS may contribute to mitochondrial damage, reduction of ΔΨm, release of cytochrome c and Smac and subsequent caspase activation and apoptosis (8,9). Apoptosis is a major control mechanism of cell death when DNA damage is not repaired. Apoptosis is a gene-directed programmed cell death characterized by cell shrinkage, blebbing of the plasma membrane, chromosomal DNA fragmentation and a number of other morphological alterations (10). There are two major pathways of apoptosis, the cell death receptor- (extrinsic) and mitochondria-mediated apoptotic (intrinsic) pathways (11). Mitochondria are central to the intrinsic apoptotic pathway. Disruption of ΔΨm, loss of mitochondrial potential and induction of cytochrome c release from the mitochondria into the cytosol...
are all associated with regulation of apoptosis and are recognized as key steps in the mitochondrion-dependent apoptotic pathway (12-14). Deltonin, diosgenin-3-O-β-D-glucopyranosyl (1→4)-α-L-rhamnopyranosyl (1→2)-β-D-glucopyranoside, is a steroidal saponin. The antiproliferative and apoptosis-inducing effects of steroidal saponins, including compounds isolated from plants and synthesized analogs, have been demonstrated in vitro using a number of cancer cell lines (15-18). We have previously demonstrated that deltonin has cytotoxic effects against colon cancer cell lines and oral administration of deltonin was found to significantly inhibit tumor growth and prolonged survival of tumor bearing mice (19,20). However, the effects of deltonin in breast cancer remain unknown. To elucidate the anticancer activity and mechanisms by which deltonin functions, the effects of deltonin on cell proliferation and apoptosis in the MDA-MB-231 human breast carcinoma cell line were evaluated. Deltonin induced apoptosis through loss of ΔΨm, accumulation of intracellular ROS, caspase activation, regulation of Bcl-2 family members and modulation of extracellular signal-regulated kinase (ERK)/AKT signaling pathways.

Materials and methods

Reagents. Deltonin was obtained as previously described and its purity determined by high performance liquid chromatography (>98%) (19). The compound was prepared as a stock solution in DMSO [final concentration <0.05% (v/v)] and diluted in the relevant culture medium. ROS assay and JC-1 ΔΨm detection kits were obtained from Beyotime Institute of Biotechnology (Jiangsu, China). 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium (MTT), DMSO, N-acetylcysteine (NAC) and primary antibody against GAPDH were purchased from Sigma-Aldrich (St. Louis, MO, USA). Primary antibodies against caspase-3 and -8, ERK1/2, phospho-ERK1/2 (Thr202/204), AKT and phospho-AKT (Ser473) were purchased from Cell Signaling Technology (Beverly, MA, USA). Primary antibodies against Bcl-2 (C-2), Bax (P19) and secondary antibodies were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). All other chemicals and reagents were of the highest purity grade commercially available.

Cell lines and cell culture. Human breast carcinoma cell line, MDA-MB-231, was obtained from the American Type Culture Collection (Manassas, VA, USA) and maintained in RPMI-1640 medium containing 10% fetal bovine serum (Gibco-BRL, Auckland, New Zealand), 100 U/ml penicillin and 100 µg/ml streptomycin in a humid chamber at 37°C under 5% CO2 (21).

Cytotoxicity assay. The inhibitory effect of deltonin on cell viability was measured by MTT assay (22). Cells were seeded in 96-well plates (Costar Corning, Rochester, NY, USA) at a density of 3x10^3 cells/well. Deltonin underwent serial dilution (0.5, 1.0, 2.0, 4.0, 8.0 µM) and DMSO (<0.05%) was used as a control. Absorbance at 570 nm was measured with Spectra Max M5 (Molecular Devices, LLC, Sunnyvale, CA, USA) following drug treatment (12, 24, 36, 48 h). All experiments were performed in triplicate. Dose- and time-dependent curves of deltonin-treated MDA-MB-231 cells were generated as the percentage cell growth inhibition, using the following formula: % inhibition = 1 - A570treated-cells/A570control-cells x 100. The 50% inhibiting concentration was calculated using SPSS software v13.0.

Flow cytometry assay for apoptosis. The use of Annexin V-fluorescein isothiocyanate (FITC) and propidium iodide (PI) for flow cytometry provides a rapid and convenient assay for apoptosis as described previously (23). Cells were exposed to desired concentrations of deltonin for 24 h and the apoptotic index was assessed using an Annexin V-FITC apoptosis kit (KeyGEN, Nanjing, China) according to the manufacturer's instructions. For each analysis, 30,000 events were acquired on a forward and side scatter gate. Annexin V-positive PI-negative cells represented early apoptotic populations and Annexin V-positive PI-positive cells represented late apoptotic or secondary necrotic populations. Data acquisition and analysis were performed by fluorescence-activated cell sorting using list mode software (Becton-Dickinson, Franklin Lakes, NJ, USA).

Measurement of ΔΨm. Alterations in ΔΨm were analyzed by flow cytometry using a ΔΨm-sensitive molecular probe dye, JC-1, as described previously (24). The JC-1 dye bearing a delocalized positive charge enters the mitochondrial matrix due to the negative charge established by the intact ΔΨm. In healthy cells, JC-1 dye stains the mitochondria red due to formation of JC-1 aggregates. In apoptotic cells, JC-1 dye accumulates in the cytoplasm in monomeric form (green fluorescence) due to collapse of ΔΨm. Cells were treated with 3.0 µM deltonin for 0, 4, 8, 12, 16 and 24 h. Following this, cells were harvested, washed once in cold PBS and incubated with JC-1 dye at 37°C for 20 min. Stained cells were washed and re-suspended in 0.5 ml assay buffer and the fluorescence was measured using flow cytometry. The emission wavelengths of JC-1 monomers and JC-1 aggregates were 530 and 590 nm, respectively.

Measurement of intracellular ROS. Intracellular ROS generation was monitored using an ROS assay kit and flow cytometry using fluorescence produced by 2',7'-dichlorofluorescein following oxidation from 2',7'- dichlorofluorescein-diacetate (DCFH-DA; Molecular Probes, Grand Island, NY, USA) (25). In brief, following treatment with 3.0 µM deltonin for 0, 4, 8, 12, 16 and 24 h, cells were harvested and then incubated with 10 µM DCFH-DA in a culture medium at 37°C for 30 min. Cells were washed and re-suspended in PBS. ROS generation was measured by flow cytometry.

Inhibitor treatment. To confirm the role of intracellular ROS in deltonin-induced apoptosis, a common quencher of ROS, NAC, was used to inhibit intracellular alteration of redox states (26). NAC was dissolved in PBS and adjusted to pH 7.4 to produce a 0.1 M stock solution. Cells were pre-incubated with 6 mM NAC for 2 h and then treated with 3 µM deltonin. After 8 h, alterations in ROS production were determined by flow cytometry. After 24 h, percentage of apoptotic cells and alterations in ΔΨm were analyzed by flow cytometry.

Protein extraction and western blot analysis. Western blot analysis was performed as described previously (27). Cells
(1x10^6/well) were grown in 6-well microplates overnight and treated with various concentrations (0, 1, 3, 5 µM) of deltonin for 24 h. Following this, cells were washed with ice-cold PBS and total cell lysates were prepared by RIPA buffer. Lysates was centrifuged at 12,000 g for 15 min at 4˚C. The supernatant was collected and total protein concentrations were determined using the BCA assay, dissolved in 5X SDS sample buffer and denatured. Proteins were separated on 10-15% SDS-PAGE and transferred to polyvinylidene fluoride membrane (Bio-Rad, Hercules, CA, USA). Membranes were incubated overnight at 4˚C with the respective primary antibodies [caspase-3 and -8, poly (ADP ribose) polymerase (PARP), Bcl-2, Bax, ERK1/2, phospho-ERK1/2, AKT and phospho-AKT] and horseradish peroxidase-conjugated secondary antibodies at 37˚C for 1 h. Reactive bands were identified using an enhanced chemiluminescent substrate to horseradish peroxidase (Amersham Pharmacia Biotech, Amersham, UK). Expression of GAPDH was used as a control.

Statistical analysis. Data are presented as mean ± SE and were analyzed for statistical significance using analysis of variance, followed by Scheffe's test for multiple comparisons. P<0.05 was considered to indicate a statistically significant difference.

Results

Deltonin decreases cell viability and induces apoptosis. The chemical structure of deltonin is presented in Fig. 1A. The cytotoxic effects of deltonin were investigated by MTT assay. As demonstrated in Fig. 1B, the maximum inhibition ratio obtained with 8 µM deltonin treatment was 89.40%. Deltonin inhibited the proliferation of MDA-MB-231 cells in a time- and concentration-dependent manner.

Flow cytometry analysis with an Annexin V-FITC apoptosis kit was also performed to identify the effects of deltonin. As shown in Fig. 2A, deltonin treatment induced apoptosis in MDA-MB-231 cells in a concentration-dependent manner. Compared with untreated cells, cells treated with 5 µM deltonin resulted in up to 75.7% apoptosis incidence, indicating that deltonin causes death in MDA-MB-231 cells by the induction of apoptosis.

Effect of deltonin on the expression of apoptosis-related proteins. Apoptosis is executed by the coordinated actions of caspase family members and is tightly regulated by Bcl-2 protein family members. Therefore, protein expression levels of these molecules were analyzed by western blot analysis. As demonstrated in Fig. 2B, treatment of MDA-MB-231 cells with deltonin led to activation of caspase-3 and -8. It is well known that activation of caspase-3 during apoptosis causes the cleavage of PARP, a major apoptotic enzyme. Deltonin increased levels of cleaved PARP in a dose-dependent manner, consistent with deltonin-induced apoptosis in MDA-MB-231 cells. Following this, the protein expression levels of Bcl-2

![Figure 1. Chemical structure of deltonin and cytotoxic effects on MDA-MB-231 cells. (A) Chemical structure of deltonin. (B) MDA-MB-231 cells were treated with various concentrations of deltonin for 12, 24, 36 and 48 h. Inhibition rate of deltonin was detected by MTT assay. Deltonin inhibited proliferation of MDA-MB-231 cells in a time- and dose-dependent manner. Values are presented as mean ± SE of three experiments. P<0.05, vs. control group. MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium.](image1)

![Figure 2. Deltonin induced apoptosis in MDA-MB-231 cells. (A) Flow cytometry analysis of Annexin V-FITC and PI staining to determine apoptosis in 24 h deltonin-treated MDA-MB-231 cells. (B) Western blot analysis of caspase-8 and -3, Bcl-2, Bax and PARP protein expression. GAPDH was used as a loading control. FITC, fluorescein isothiocyanate; PI, propidium iodide; PARP, poly (ADP ribose) polymerase.](image2)
family members, Bcl-2 and Bax were determined. Fig. 2B shows a decrease in Bcl-2 and increase in Bax levels.

**Effect of deltonin on ΔΨm change.** Change of ΔΨm using JC-1 was performed to investigate whether mitochondria are involved in deltonin-induced apoptosis. Representative flow cytometric results are presented in Fig. 3. Deltonin treatment led to a time-dependent increase in the number of green fluorescence-positive cells from 9.5 in untreated cells to 17.1, 23.3, 40.9, 49.3 and 58.6%.

**Effect of deltonin on intracellular ROS.** ΔΨm loss is an early event in apoptosis induction, which is often accompanied by ROS production. Intracellular ROS was examined using DCFH-DA to analyze the role of ROS in deltonin-induced apoptosis. As demonstrated in Fig. 4, treatment of MDA-MB-231 cells with 3 µM deltonin for 0, 4, 8, 12, 16 and 24 h resulted in ROS increase from 10.3 to 66% from 0 to 8 h, however, ROS levels decreased to 13.7% at 24 h. These results revealed that the ROS burst is generated in a time-dependent manner.

**NAC inhibits deltonin-induced apoptosis.** To determine the association between intracellular ROS and deltonin-induced apoptosis, MDA-MB-231 cells were pretreated with 6 mM NAC for 2 h and then incubated with 3 µM deltonin. As revealed in Fig. 5A, pretreatment with NAC reduced the number of apoptotic cells, whereby the percentage of apoptotic cells decreased from 32.9 to 23.4%.

**NAC inhibits deltonin-induced intracellular ROS accumulation.** NAC markedly inhibited deltonin-induced accumulation of ROS, the intensity of DCFH-DA fluorescence decreased from 54.8 to 18.0% (Fig. 5B).

**NAC inhibits deltonin-induced depolarization of ΔΨm.** NAC is known to exert protective effects on cells, therefore, the ability of NAC to prevent depolarization of ΔΨm in deltonin-treated cells was investigated. Fig. 5C reveals flow cytometry analysis of ΔΨm in the presence of absence of NAC (6 mM) pretreatment. The results demonstrate that deltonin-induced apoptosis in MDA-MB-231 cells may be
mediated by the mitochondrial pathway and, at least in part, by accumulation of ROS.

**Effect of deltonin on the activation of ERK1/2 and AKT.** ERK and AKT are two notable pathways associated with tumor development. Inhibition of the ERK pathway or AKT activation has been identified to induce apoptosis in tumor cells (28-30). To investigate the involvement of ERK/AKT signal pathways in deltonin-induced apoptosis of MDA-MB-231 cells, levels of phosphorylated and unphosphorylated AKT and ERK1/2 were determined by western blot analysis. As shown in Fig. 6, treatment of deltonin decreased levels of phospho-ERK1/2 and -AKT in a dose-dependent manner compared with GAPDH (loading control). Results indicate that interference with ERK and AKT signal pathways may contribute to the anticancer activity of deltonin.

**Discussion**

*Dioscorea zingiberensis* Wright, a widely distributed medicinal plant, has been used extensively for a number of years in China. Deltonin is a major component of steroidal saponins from *Dioscorea zingiberensis* Wright and it has been reported to exhibit anticancer properties which may prove suitable for cancer therapy. In the present study, the effect of deltonin on breast cancer cells was evaluated and deltonin was found to significantly inhibit the growth of human breast cancer MDA-MB-231 cells in a time- and dose-dependent manner. Apoptosis is a form of programmed cell death which occurs in response to a variety of stimuli and is characterized by a series of morphological and biochemical changes. Apoptosis is divided into two signaling pathways, death receptor-mediated (extrinsic) and mitochondrial (intrinsic) (31). Caspases are central to the majority of apoptotic responses. Activation of caspase-3 is considered to initiate apoptosis and requires activation of initiator caspases, including caspase-8 or -9, in response to proapoptotic signals (32,33). In the apoptotic assay, results of flow cytometry analysis confirmed that the percentage of apoptotic cells increased with deltonin concentration. In addition, the induction of apoptosis was confirmed by western blot analysis. Treatment of MDA-MB-231 cells with deltonin led to the degradation of caspase-3 and -8 and cleavage of PARP in a dose-responsive manner. Bcl-2 family members are recognized as important regulators of apoptosis. In the current study, the expression levels of key Bcl-2 family proteins were determined. Western blot analysis demonstrated that deltonin downregulated Bcl-2 levels and increased levels of Bax. Bcl-2 is an important regulator of apoptosis by control of mitochondrial function. Bcl-2 affects the release of cytochrome c from the mitochondria and activates a number of caspases (34,35). Therefore, additional analysis focused on the mitochondrial pathway was performed.

Mitochondrial changes, including permeability transition pore opening and the collapse of ∆Ψm, induces apoptosis by activation of caspases (36). In the present study, the treatment of MDA-MB-231 cells with deltonin resulted in the loss of ∆Ψm in a time-dependent manner. The results indicate that deltonin induced apoptosis of MDA-MB-231 cells, at least in part, through the mitochondrial damage-mediated caspase pathway.
The role of ROS in mediating apoptosis in various cancer cells is well established (37). ROS generation causes functional disorder of mitochondria and leads to cell apoptosis (38,39). Results indicate that deltonin induced generation of ROS and collapse of ∆Ψm. Intracellular ROS levels were observed to significantly increase in a time-dependent manner and peaked at 8 h of deltonin treatment, indicating that deltonin may result in accumulation of intracellular ROS. To determine whether increased production of ROS was critical for deltonin-induced apoptosis, cells were pretreated with the ROS scavenger, NAC. Results revealed that NAC had a suppressive effect on deltonin-induced apoptosis, intracellular ROS generation and depolarization of ∆Ψm, indicating that ROS is associated with activation of the mitochondrial pathway. In general, results demonstrate that deltonin induces apoptosis by a mechanism involving increased intracellular ROS levels in MDA-MB-231 cells.

AKT signaling is crucial for initiation and progression of breast cancer (40) and also regulates several downstream targets responsible for cell survival and proliferation (41-44). In the current study, deltonin downregulated expression of phospho-AKT, indicating that deltonin inhibits AKT-mediated survival signaling in breast cancer cells. In addition, the ERK signaling pathway is an important pathway associated with the survival signaling in breast cancer cells. In addition, the ERK1/2 phosphorylation was significantly decreased by deltonin treatment, suggesting that deltonin inhibits AKT-mediated survival signaling in breast cancer cells. In addition, the ERK1/2 signaling pathway is an important pathway associated with cell survival and growth. In the present study, deltonin treatment altered the expression of phospho-ERK1/2 in MDA-MB-231 cells, demonstrating that deltonin-induced apoptosis of MDA-MB-231 cells is associated with inhibition of phosphorylation of AKT and ERK1/2.

In conclusion, our results demonstrate that the cytotoxic effect of deltonin in MDA-MB-231 cells is mediated by the induction of apoptosis. Deltonin-induced apoptosis involves the regulation of Bcl-2 family members and caspase-dependent mitochondrial dysfunction and also appears to be markedly associated with ROS production and regulation of ERK and AKT signaling pathways. Results indicate that deltonin may be an effective therapeutic agent for the treatment of breast cancer.

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References


