Deltonin Isolated from *Dioscorea zingiberensis* Inhibits Cancer Cell Growth through Inducing Mitochondrial Apoptosis and Suppressing Akt and Mitogen Activated Protein Kinase Signals

Dan Shu, Yong Qing, Qingyi Tong, Yang He, Zhihua Xing, Yinglan Zhao, Yi Li, Yuquan Wei, Wen Huang, and Xiaohua Wu

*Laboratory of Ethnopharmacology, Regenerative Medicine Research Center, State Key Laboratory of Biotherapy, West China Hospital, Sichuan University; No. 1, Keyuan 4lu, Gaopeng Avenue, Gaoxinqu, Chengdu 610041, China.*

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Deltonin is an active component purified from *Dioscorea zingiberensis* Wright (DZW), and has shown anticancer effects. However, its mechanism of action remains elusive. In the present study, we investigated the effect of Deltonin on a panel of cancer cell lines and analyzed its mechanism in C26 cells, a murine colon carcinoma cell. Our results showed that Deltonin markedly inhibited the growth of all examined cancer cell lines. Deltonin induced dose- and time-dependent apoptosis in C26 cells. The event of apoptosis was accompanied by the release of cytochrome c, depolarization of mitochondrial membrane potential, and dose- and time-dependent reactive oxygen species (ROS) generation. Deltonin also increased the expression of Bax, decreased the expression of B-cell lymphoma/lewkmia-2 (Bcl-2), and induced the activation of caspase 9, caspase 3 and poly(ADP-ribose) polymerase (PARP). Furthermore, Deltonin decreased Akt and extracellular signal-regulated kinase-1/2 (ERK1,2) activity. These results demonstrate that Deltonin mediates the growth inhibition of cancer cells through multiple targets, which include the generation of reactive oxygen species (ROS), mitochondrial apoptosis and the inhibition of the mitogen-activated protein kinase (MAPK) and Akt signaling pathways, suggesting Deltonin is a potent cancer preventive and therapeutic agent.

Key words  Deltonin; apoptosis; mitochondria; reactive oxygen species; Akt; mitogen-activated protein kinase

The high efficiency and lower toxicity of natural products have made them great candidates to be explored for cancer treatment. Steroidal saponins from a variety of plants have shown various biological activities, including anticancer effects, and several steroidal saponins such as Paris saponin I isolated from *Rhizoma paridis* and Dioscin from varied Dioscorea plants, have been widely studied and shown to exert anticancer activity by the induction of apoptosis via different signaling pathways. In *Dioscorea zingiberensis* Wright (DZW), a widely distributed medicinal plant in China. Its rhizome has been used as a traditional Chinese medicine for a long time and used as a folk treatment for cough, anthrax, rheumatic heart disease, rheum, arthritis, tumefaction and sprain. Steroidal saponins are the major active components of DZW, and Deltonin, diosgenin-3-O-β-D-glucopyranosyl(1→4)-[α-L-rhamnopyranosyl(1→2)→β-D-glucopyranoside, is one of the main constituents of these steroidal saponins. In our previous study, we found that Deltonin was effective in the treatment of cancer cells and was able to induce cancer cell apoptosis both in *vitro* and *in vivo*. In the present study, we investigated the anticancer effect of Deltonin on a panel of cancer cell lines and analyzed its mechanism in C26 cells. We found that Deltonin mediates the growth inhibition of cancer cells through mitochondrial apoptosis, reactive oxygen species (ROS) generation, Akt and mitogen-activated protein kinase (MAPK) pathways.

**MATERIALS AND METHODS**

**Chemical Reagents and Antibodies**  The fresh rhizomes of DZW were supplied by Tianhe Pharmaceutical Company, Yunxi, Hubei, China. They were authenticated by the Hubei Province Institute of Drug Identification. The Deltonin was isolated from the rhizomes of DZW using silica gel, macroporous adsorption resin, Sephadex LH-20, RP-C18 column chromatography and a vacuum system (Büchi, Switzerland). Its structure, diosgenin-3-O-β-D-glucopyranosyl(1→4)-[α-L-rhamnopyranosyl(1→2)]-β-D-glucopyranoside, was identified on the basis of its spectral data (1D- and 2D-NMR, MS and IR experiments) and its purity as determined by HPLC was above 98%. The Deltonin was dissolved in dimethylsulfoxide (DMSO) prior to use. The final concentration of DMSO was under 0.1% (v/v) throughout the experiments. 3-[4,5-Dimethylthiazol-2-yl]-2,5-diphenyltetrazolium (MTT) and 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium (MTT) and Rnase A were purchased from Sigma (St. Louis, MO, U.S.A.). 4′,6′-Diamidin-2′-phenylindol-dihydrochloride (DAPI) was purchased from Roche Diagnostics GmbH (Mannheim, Germany). DNA marker and Proteinase K were purchased from TakaRa (Dalian, China). Cell Mitochondria Isolation Kit, Reactive Oxygen Species Assay Kit and JC-1 Mitochondrial Membrane Potential Detection Kit were purchased from Beyotime (Jiangsu, China). The primary antibodies were obtained as follows: antibodies for caspase 3, caspase 9 and poly (ADP-ribose) polymerase (PARP), ERK1,2, phospho-ERK1,2 (Thr202/Tyr204), Akt, phospho-Akt (Ser473) were purchased from Cell Signaling Technology (Beverly, MA, U.S.A.); Antibodies for B cell lymphoma/lewkmia-2 (Bcl-2), Bax, β-actin and the secondary antibodies, which were horseradish peroxidase (HRP)-linked goat anti-mouse immunoglobulin (IgG) and goat anti-rabbit IgG, were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, U.S.A.); Antibody for cytochrome c was purchased from Abcam. Other reagents were obtained in the highest purity grade available commercially.

*To whom correspondence should be addressed.  e-mail: wuxh@scu.edu.cn; huangwen@scu.edu.cn  These authors contributed equally to this work.*
Cell Culture  HEK293, a human embryonic kidney cell line; A549, a human lung adenocarcinoma cell line; MDA-MB-231, a breast adenocarcinoma cell line; LL/2, a Lewis Lung carcinoma cell line; SKOV3, an ovary adenocarcinoma cell line; B16, a melanoma adenocarcinoma cell line; PC-3, a prostate adenocarcinoma cell line; and C26, a colon adenocarcinoma cell line, were obtained from the American Type Culture Collection. Cells were cultured and maintained in RPMI 1640 or Dulbecco’s modified Eagle’s medium (DMEM) medium (Gibco BRL, Grand Island, NY, U.S.A.) supplemented with 10% heat inactivated fetal bovine serum (FBS, Gibco, Auckland, New Zealand), 100 U of penicillin G and 100 U of streptomycin sulfate under 5% CO₂ and 95% humidified air atmosphere at 37°C.

MTT Assay for Cell Viability  The effect of Deltonin on cell viability was determined using MTT assay, as described previously. Briefly, 2 × 10⁵ cells/well were plated in 96-well plates (Costar Corning, Rochester, NY, U.S.A.). After treatment with various concentrations of Deltonin (0, 0.1, 0.2, 0.4, 0.8, 1.6, 3.2 μM) for 48 h on all the cell lines or treatment with 2.0 μM for 0, 6, 12, 24, 48, 72 h on the C26 cells. The cells were treated with 20 μl of 5 mg/ml MTT and the resulting formazan crystals were dissolved in dimethylsulfoxide (200 μl). Absorbance was recorded at 570 nm with a reference at 650 nm serving as the blank. The effect of Deltonin on cell viability was assessed as the percent cell viability compared to the control cells, which were arbitrarily assigned 100% viability. The 50% inhibiting concentration (IC₅₀) was calculated by Regression Probit Analysis (SPSS software version 13.0).

Morphological Analysis  Approximately 2 × 10⁵ C26 cells were seeded in each well of 6-well plates. After incubation overnight, Deltonin (0, 1.6, 3.2 μM) was added, and the cells were then incubated at 37°C for 48 h. Untreated or Deltonin-treated C26 cells were examined for morphological changes using an inverted fluorescence microscope (Olympus, Tokyo, Japan). For identification of the apoptotic cells, the cells were stained with DAPI and incubated at 37°C for 15 min, washed with PBS and observed with an inverted fluorescence microscope (Zeiss, Axiovert 200, Germany).

Analysis of DNA Fragmentation  Cleavage of DNA at the internucleosomal linker sites yielding DNA fragments in multiples fragments (180—200 bp) was a biochemical hallmark of apoptosis. Agarose gel electrophoresis was performed for analysis of DNA fragmentation. Briefly, cells (5 × 10⁶) were lysed with 0.5 ml lysis buffer containing 5 mM Tris/HCl (pH 8.0), 0.25% Nonidet P-40, and 1 mM ethylene diamine tetraacetic acid (EDTA), followed by the addition of RNase A at a final concentration of 200 μg/ml, and incubated for 1 h at 37°C. Cells were then treated with 400 μg/ml Proteinase K for another hour at 37°C. After the addition of 4 μl loading buffer, 20 μl samples in each lane were subjected to electrophoresis on 1.5% agarose at 50 V for 2 h. DNA was stained with ethidium bromide. After electrophoresis, the DNA bands were visualized with images captured under UV light using the Bio-Rad VersaDoc Imaging System.

Annexin V-Fluorescein Isothiocyanate (FITC) Binding Assay  To quantify the Deltonin-induced apoptotic death of C26 cells, Annexin V and PI staining was performed by flow cytometry, as described previously. Both floating and attached cells were collected by brief trypsinization and washed with phosphate buffered saline (PBS) twice, then subjected to Annexin V and PI staining using an Apoptosis Assay Kit (Keygentec, Nanjing, China) following the step-by-step protocol provided by the manufacturer. After staining, the quantification of apoptotic cells was measured with a Becton-Dickinson FACS-Calibur flow cytometer.

Detection of Cytochrome c Release from Mitochondria  At the end of Deltonin treatment, C26 cells were washed with ice-cold PBS and collected by a brief trypsinization followed by two more washes with PBS. The cell pellet was re-suspended in 200 μl of extraction buffer containing 210 mM mannitol, 70 mM sucrose, 20 mM HEPES-KOH, pH 7.4, 50 mM KCl, 5 mM ethylene glycol-bis(2-aminoethyl)-N,N,N’,N’-tetraacetic acid (EGTA), 2 mM MgCl₂, 1 mM dithiothreitol, 0.1 mM phenylmethylsulfonyl fluoride (PMSF) and protease inhibitors (Complete Cocktail; Roche Molecular Biochemicals, Indianapolis, IN, U.S.A.). After 30 min incubation on ice, cells were homogenized with a glass Dounce and pestle, and the resulting homogenate was left on ice for an additional 20 min. Homogenates were centrifuged at 600 × g for 15 min at 4°C, and the resulting supernatant was further centrifuged at 11000 × g for 15 min at 4°C, to yield cytosolic extract, 40 μg protein per sample, which was resolved on 15% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto a nitrocellulose membrane, followed by blocking in 5% (w/v) non-fat dry milk in TBS. The membrane was probed with an anti-cytochrome c antibody overnight at 4°C followed by 1 h incubation with HRP-conjugated secondary antibody. To ensure equal protein loading, each membrane was stripped and reprobed with anti-β-actin antibody.

Measurement of Mitochondrial Membrane Potential (ΔΨₘ)  The loss of mitochondrial membrane potential (ΔΨₘ) was quantitatively determined by flow cytometry using the lipophilic cationic probe JC-1 dye (5,5’,6,6’-tetra-chloro-1,1’,3,3’-tetraethylbenzimidazolocarbocyanine iodide) Detection Kit. The JC-1 dye bearing a delocalized positive charge enters the mitochondrial matrix due to the negative charge established by the intact mitochondrial membrane potential. In healthy cells, JC-1 dye stains the mitochondria red due to the formation of JC-1 aggregates. In apoptotic cells, JC-1 dye accumulates in the cytoplasm in monomeric form (green fluorescence) due to the collapse of the mitochondrial membrane potential. Briefly, cells were treated with 2.0 μM doses of Deltonin for 0, 6, 12, 16, 20 and 24 h. The cells were incubated with JC-1 dye (10 μg/ml in PBS) at 37°C for 20 min. Cells were washed and re-suspended in 0.5 ml assay buffer and the fluorescence was measured using a Becton-Dickinson FACS-Calibur flow cytometer. The emission wavelengths of JC-1 monomers and JC-1 aggregates were respectively ca. 530 nm and ca. 590 nm.

Measurement of Reactive Oxygen Species (ROS) Using Flow Cytometry  ROS production was monitored by flow cytometry using 2’,7’-dichlorodihydrofluorescein diacetate (DCFH-DA). DCFH-DA, a non-fluorescent cell-membrane permeable probe, was used to penetrate the cells, which were then reacted with cellular ROS, and metabolized into fluorescent DCF. After treatment with 2.0 μM of Deltonin for 0, 6, 12, 16, 20 and 24 h or treatment with Deltonin (0, 0.8, 1.6, 3.2 μM) for 6 h and 12 h, cells were trypsinized and collected...
in ice cold PBS. Cells were incubated with 10 \( \mu M \) DCFH-DA in a culture medium at 37°C for 30 min. Cells were collected by centrifugation, washed and resuspended in PBS. The intracellular ROS, as indicated by dichlorofluorescein (DCF) fluorescence, was measured with a flow cytometer.

**Western Blotting Analysis** According to the methods described previously, protein expression in the C26 cells after Deltonin treatment at different concentrations and time intervals was analyzed by Western blotting. C26 cells (1 \( \times \) 10^6 cells per 25 cm² flask) were treated with Deltonin at various concentrations (0, 0.8, 1.6, 3.2 \( \mu M \)) for 48 h and 2.0 \( \mu M \) for 0, 4, 8, 16, 24, 48 h. Proteins were extracted on ice in 400 \( \mu l \) of modified RIPA buffer, which containing 50 mm Tris–HCl (pH 7.4), 1% NP-40, 0.25% Na-deoxycholate, 150 mm NaCl, 1 mm pheny methy sulphonyl fluoride, 1 mm Na2VO4, 1 mm NaF and a protease inhibitor cocktail. The total proteins were quantified using a coomassie protein assay reagent. The proteins were separated according to molecular weight on a 10—15% SDS–PAGE gel and transferred onto a polyvinylidene fluoride (PVDF) membrane (Bio-Rad, Hercules, CA, U.S.A.). Membranes were blocked with 5% nonfat milk in Tris-Buffered Saline Tween-20 (TBST) at room temperature for 1 h, and probed with specific primary antibodies overnight at 4 °C. After washing, the blots were incubated with antibodies detected using either a goat-anti-mouse or goat-anti-rabbit horseradish peroxidase-conjugated secondary antibody. Blots were washed thrice for about 5 min with TBST and once for 5 min with TBS, followed by a 1 min incubation with enhanced chemiluminescent substrate and exposure to Kodak X-OMAT autoradio-graphic film (Kodak, Hemel Hempstead, United Kingdom).

**Statistical Analysis** Differences were evaluated by Student’s \( t \)-test or one-way ANOVA followed by Dunnett’s multiple comparison tests. A \( p \) value of < 0.01 or < 0.05 was regarded as indicating a significant difference.

**RESULTS**

**Effects of Deltonin on Cell Viability** The chemical structure of Deltonin is shown in Fig. 1, with a molecular weight 884. To assess the effect of Deltonin on cell viability, a panel of tumor cell lines that included A549, MDA-MB-231, LL/2, SKOV3, B16, PC-3, and C26 were cultured with a range of Deltonin doses (0, 0.1, 0.2, 0.4, 0.8, 1.6, 3.2 \( \mu M \)) for 48 h. Cell viability of the C26 cells was analyzed using MTT assay. A significant reduction of cell viability was observed in a dose-dependent manner (Fig. 2A). However, we found Deltonin had less effect on the cell viability of non-tumor cells compared to tumor cells (Fig. 2A). The IC_{50} of A549, MDA-MB-231, LL/2, SKOV3, B16, PC-3, and C26 cell lines are shown in Table 1. Treatment of C26 cells with 2.0 \( \mu M \) of Deltonin for 0, 6, 12, 24, 48, 72 h produced a significant reduction in cell viability in a time-dependent manner (Fig. 2B). These results indicate that Deltonin inhibits the proliferation of C26 cells in a dose- and time-dependent manner.

**Deltonin Induces Apoptosis in C26 Cells** To determine whether Deltonin inhibits cell viability due to apoptosis, morphological observation, DNA fragmentation and Annexin V binding of C26 cells were examined. We treated C26 cells with Deltonin of 0, 1.6 and 3.2 \( \mu M \) for 48 h, and the nuclear morphology of the cells was observed by DAPI staining. As shown in Fig. 3Af, condensed and larger nuclei were deep white, as seen in cells after treatment with 3.2 \( \mu M \) of Deltonin for 48 h, whereas the non-Deltonin-treated cells showed normal round and small nuclei, as seen in Fig. 3Ad. One of the most characteristic phenomena of apoptosis is the activation of endonucleases leading to the fragmentation of the genomic DNA.17) We determined DNA fragments by agarose gel electrophoresis. As shown in Fig. 3B, Deltonin at a dose of 3.2 \( \mu M \) caused significant DNA fragmentation. We also quantified apoptosis by FACS using an AnnexinV-FITC Apoptosis Assay Kit. As shown in Fig. 3C, treatment of C26

<table>
<thead>
<tr>
<th>Tumor cell lines</th>
<th>IC_{50} (( \mu M ))</th>
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<tbody>
<tr>
<td>A549</td>
<td>3.09±0.79</td>
</tr>
<tr>
<td>MDA-MB-231</td>
<td>1.58±0.09</td>
</tr>
<tr>
<td>LL/2</td>
<td>1.80±0.19</td>
</tr>
<tr>
<td>SKOV3</td>
<td>1.32±0.15</td>
</tr>
<tr>
<td>B16</td>
<td>2.59±1.04</td>
</tr>
<tr>
<td>PC-3</td>
<td>2.31±0.38</td>
</tr>
<tr>
<td>C26</td>
<td>1.21±0.09</td>
</tr>
</tbody>
</table>
cells with Deltonin in 0, 0.8, 1.6 and 3.2 \( \mu M \) concentration resulted in a dose-dependent increase in the number of total apoptotic cells, from 10.3 to 24.9\%, 56.6\% and 98\%, respectively. These results suggest that the induction of apoptosis may be a major mechanism of the Deltonin-caused inhibition of the viability of C26 cells.

**Deltonin Affects the Cytochrome \( c \) Release and Mitochondrial Membrane Potential**

The release of cytochrome \( c \) from mitochondria into cytosol is one of the early events leading to apoptosis. To examine whether Deltonin could induce cytochrome \( c \) release from mitochondria into cytosol, we treated C26 cells with Deltonin of 0, 0.8, 1.6 and 3.2 \( \mu M \) for 12 h or 24 h, and the result with 24 h of Deltonin treatment showed a dose-dependent increase in the release of cytochrome \( c \) into the cytoplasm (Fig. 4A). However, at 12 h with Deltonin treatment, we could not find any expression of cytochrome \( c \) in the cytoplasm (data were not shown).

Release of cytochrome \( c \) into the cytosol is usually preceded or accompanied by a drop in \( \Delta \Psi_m \). Therefore, we determined whether Deltonin induces a loss or disruption of mitochondrial membrane potential in C26 cells. C26 cells were treated with 2.0 \( \mu M \) of Deltonin for 0, 6, 12, 16, 20 and 24 h, and the result with 24 h of Deltonin treatment showed a dose-dependent increase in the release of cytochrome \( c \) into the cytoplasm (Fig. 4A). However, at 12 h with Deltonin treatment, we could not find any expression of cytochrome \( c \) in the cytoplasm (data were not shown).

**Deltonin Increases Generation of ROS**

\( \Delta \Psi_m \) loss is an early event in apoptosis induction, which is often accompanied by the production of reactive oxygen species. To determine whether the reduction of mitochondrial membrane depolarization related to the generation of ROS, we treated C26 cells with Deltonin (0, 0.8, 1.6, 3.2 \( \mu M \)) for 6 h and 12 h, and the generation of ROS levels at 6 h and 12 h increased in a dose-dependent manner. As shown in Fig. 5C, the 6 h result of cell fluorescence intensity was 1.6\%, 2.9\%, 6.5\%, 18.4\%, respectively with 0, 0.8, 1.6, 3.2 \( \mu M \) of Deltonin, and the fluorescence intensity at 3.2 \( \mu M \) increased more than 10-fold as compared to the non-Deltonin-treated cells (data of 12 h were not shown). These results indicate that apoptosis induced by Deltonin is associated with the generation of ROS.

**Deltonin Regulates Expression of Bcl-2 and Bax, Induces Activation of Caspases, and PARP**

To further de-
termine the apoptotic pathway involved in the response of cells to Deltonin treatment, we examined the components of the apoptotic pathway. Increased levels of the pro-apoptotic protein Bax and a dramatic reduction in anti-apoptotic protein Bcl-2 were observed with Deltonin treatment (Fig. 6). Treatment of C26 cells for 48 h with Deltonin (0, 0.8, 1.6, 3.2 \( \mu \)M) also resulted in a dose-dependent activation of caspase 9, caspase 3 and PARP when compared with untreated cells (Fig. 6). Collectively, these results suggest that Deltonin induced apoptosis in C26 cells is mediated through the activation of caspases.

**Deltonin Inhibits Phosphorylation of ERK 1/2 and Akt in C26 Cells** ERK\(_{1/2}\) and Akt are two important pathways associated with tumor development. The inhibition of either the MAPK pathway or Akt activation has been shown to induce apoptosis in tumor cells.\(^{20-22}\) Hence, to determine whether Deltonin inhibits the growth of C26 cells and simultaneously promotes apoptosis by modulating the two pathways, we observed changes in the phosphorylation of ERK\(_{1/2}\) and Akt after Deltonin treatment. With different concentrations of Deltonin (0, 0.8, 1.6, 3.2 \( \mu \)M) for 48 h, we observed a dose-dependent decrease of phosphorylation of both ERK\(_{1/2}\) and Akt (Fig. 7A). Treatment of C26 cells with 2.0 \( \mu \)M of Deltonin for 0, 4, 8, 16, 24 and 48 h, the expression of total ERK\(_{1/2}\) and Akt did not show any significant alteration in comparison with \( \beta \)-actin as a loading control, whereas the expression levels of phosphorylated ERK\(_{1/2}\) (Thr202/Tyr204) and phosphorylated Akt (Ser473) were markedly decreased, especially at 48 h of Deltonin treatment (Fig. 7B). These results indicate that interference with ERK\(_{1/2}\) and Akt signal pathways may contribute to the anti-cancer activity of Deltonin.

**DISCUSSION**

Chinese traditional herbs are a potential source for identifying new anticancer compounds and a source of alternative cancer therapy.\(^{23-25}\) Deltonin, a steroidal saponin isolated from DZW, has been proved by our group to have anticancer effect.\(^{19}\) In the present study, we found that Deltonin exerted marked growth inhibition on several tumor cell lines, and had less effect on the cell viability of non-tumor cells. Further studies on C26 cells by morphological observation, DNA fragmentation assay and Annexin V binding indicate that Deltonin can induce significant apoptosis in a dose-dependent manner. Deltonin also induced the release of cyto-
The results suggest that Deltonin may be a potent chemotherapeutic agent against colon cancer by inducing apoptosis via a mitochondrial pathway. Furthermore, we also observed that Deltonin may inhibit cancer growth by interfering with ERK1/2 and Akt pathways.

Apoptosis is a biological process in cell development, tissue homeostasis, and regulation of the immune system. Inadequate apoptosis is an important cause of cancer development, and the induction of apoptosis has been a major mechanism of anticancer drugs. Apoptosis includes two major pathways. One is a receptor-mediated extrinsic pathway and the other is the mitochondria-apoptosome-mediated intrinsic pathway which involves the loss of mitochondrial membrane potential and subsequently leads to the release of cytochrome c from mitochondria into the cytosol. In mitochondrial apoptosis, the release of cytochrome c from mitochondria into the cytosol is one of the early events leading to apoptosis. As the levels of cytochrome c increase in cytosol, it interacts with Apaf-1 to form a complex with pro-caspase 9, leading to the activation of caspase 9 and caspase 3 which leads to the cleavage of PARP.

In our experiment, treatment of C26 cells with Deltonin (0, 0.8, 1.6, 3.2 μM) for 24 h showed that Deltonin induced a dose-dependent increase in cytochrome c in cytosol (Fig. 4A). As shown in Fig. 4B, ΔΨm of Deltonin-treated C26 cells for 24 h with 2.0 μM exhibited an obvious reduction compared to the non-Deltonin-treated cells. These results suggest an involvement of cytochrome c release from mitochondria, as well as a possible depolarization of mitochondrial membrane potential in Deltonin-induced apoptosis in C26 cells.

The Bcl-2 family of proteins have emerged as critical regulators of mitochondria-mediated apoptosis. The anti-apoptotic Bcl-2 family members, Bcl-2 and Bcl-xL, mainly prevent the release of apoptogenic molecules (cytochrome c) from mitochondria into the cytosol. On the other hand, the pro-apoptotic Bcl-2 family of proteins, such as Bax and Bid, induce mitochondrial outer membrane permeabilization and release of cytochrome c. An increase in the levels of pro-apoptosis proteins and/or a decrease in anti-apoptosis proteins can lead to a decrease in mitochondrial membrane potential and an opening of mitochondrial permeability transition pores, leading to cytochrome c release from mitochondria into cytosol. In the present study, we found that the treatment of C26 cells with Deltonin resulted in a dose-dependent decrease in the levels of anti-apoptotic proteins Bcl-2 and a simultaneous increase in pro-apoptotic protein Bax (Fig. 6). This alteration was responsible for the concomitant execution phase of apoptosis which included the disruption of mitochondrial membrane potential, ROS generation, and activation of caspase 9, caspase 3 and PARP.
of mitochondrial membrane potential and increased the release of cytochrome c into cytosol (Fig. 4), and also led to the activation of caspase 9, caspase 3 and PARP (Fig. 6), and ultimately cell death.

Mitochondria is a major site of ROS production. Reduction in ΔΨm was reported to be accompanied by the production of ROS in the early stages of apoptosis. However, in this study, we found that the Deltonin induced generation of ROS was preceded by changes in ΔΨm and in the release of cytochrome c. With 2.0 μM of Deltonin treatment from 0 to 24 h, the ROS levels increased continuously from 6 to 12 h, achieved a peak at 12 h, and decreased at 24 h (Fig. 5A). Deltonin also induced a dose-dependent increase in ROS levels. In C26 cells treated with 0.8, 1.6 and 3.2 μM of Deltonin for 6 h, fluorescence intensity was increased by about 1.8-, 4.1-, and 11.5-fold, respectively, compared with untreated cells (Fig. 5C). However, we could not determine changes in ΔΨm or the release of cytochrome c until 24 h of Deltonin treatment (Figs. 4A, B). Generation of ROS has been associated with the apoptotic response induced by various chemotherapeutic agents. Currently, we do not know whether the mitochondrial-dependent apoptosis induced by Deltonin is mediated by ROS, nor the exact mechanism by which Deltonin induces the generation of ROS in the C26 cells. Further work is needed to explore these mechanisms.

The Akt and MAPK signaling pathways are two critical pathways involved in cell survival, proliferation, differentiation and tumor growth. Hyperactivation of Akt and/or ERK is associated with increased cell growth, cell proliferation, metastasis, angiogenesis, and cellular energy metabolism and resistance to apoptosis, and is commonly detected.
in colon and other cancers.\textsuperscript{45–50} In this study, we found Deltonin was able to inhibit the activation of both ERK\textsubscript{1/2} and Akt signaling in a dose- and time-dependent manner (Fig. 7). The strong inhibition by Deltonin of these two signals implies to us that, besides inducing apoptosis, Deltonin may inhibit cancer cell growth by other aspects which involve Akt and ERK\textsubscript{1/2} signals.

Currently, targeting intracellular signaling pathways has been a novel strategy for drug discovery. Several multi-target kinase inhibitors, such as imatinib and sorafenib, have been in clinical use for cancer therapy.\textsuperscript{51,52} Although the mechanisms and effects of Deltonin on cancer therapy need further clarification in colon and other cancers, the present results have proved that Deltonin exerts its anti-cancer effects through multiple targets, which include the generation of ROS, inhibition of ERK and Akt signaling pathways, and direct mitochondrial apoptosis, suggesting Deltonin may have important potential as a cancer preventive and therapeutic agent.

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