Triptolide inactivates Akt and induces caspase-dependent death in cervical cancer cells via the mitochondrial pathway

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Abstract. Triptolide, the main active component of the traditional Chinese herbal medicine *Tripterygium wilfordii* Hook F, has been shown to have potent immunosuppressive and anti-inflammatory properties. Here, we investigated the pro-apoptotic effect of triptolide in human cervical cancer cells and its underlying mechanisms. Exposure of cervical cancer cells to triptolide induced apoptosis, which was accompanied by loss of mitochondrial membrane potential, caspase processing (caspase-8, -9 and -3), and cleavage of the caspase substrate, poly(ADP-ribose) polymerase. The cytotoxic effects of triptolide were significantly inhibited by the caspase inhibitor, z-VAD-fmk. Triptolide-induced apoptosis was associated with a marked reduction in Akt phosphorylation and was exacerbated by LY294002 (phosphatidylinositol-3'-kinase inhibitor). Conversely, it was attenuated by Akt overexpression. Triptolide-induced apoptosis was also associated with downregulation of Mcl-1 and was significantly inhibited by Mcl-1 overexpression. These findings show that triptolide induces caspase-dependent, mitochondria-mediated apoptosis in cervical cancer cells, in part, by negatively regulating Akt and Mcl-1.

Introduction

Triptolide, a highly oxygenated diterpene isolated from *Tripterygium wilfordii* Hook F, has been used in traditional Chinese medicine for centuries. Several studies have shown that triptolide has various activities and can function as an immunosuppressor, an anti-cancer drug, and a contraceptive (1-3). In many types of tumor cells, triptolide has been shown to induce apoptosis by promoting the release of cytochrome c as a result of mitochondrial damage and by decreasing the expression of anti-apoptotic proteins (4). Apoptosis, a highly regulated form of cell death, is distinguished by the activation of a family of caspases that cleave various proteins, resulting in characteristic morphological and biochemical changes (5-8). Upon induction of apoptosis, several molecules are released from mitochondria into the cytosol, where they perform their pro-apoptotic functions. One such protein is cytochrome c. Upon its release from mitochondria, cytochrome c binds to the adaptor protein, Apaf-1, leading to Apaf-1 oligomerization and the activation of caspases (i.e., the apoptotic proteases) (9).

A key element of the Bcl-2 mechanism is to control the permeability of the outer membrane of the mitochondria, permitting the release of regulatory factors (e.g., cytochrome c) that are located in the intermembrane space. Bcl-2 family proteins share one or more Bcl-2 homology (BH) domains and are divided into two main groups based on their pro- or anti-apoptotic activities. The anti-apoptotic members include Bcl-2, Bcl-xL and Mcl-1. The pro-apoptotic members can be further classified according to whether they contain multiple BH domains (e.g., Bax and Bak) or only the BH3 domain (e.g., Bid and Bim). The BH3 domain-only proteins require the cooperation of other multidomain family member proteins to induce apoptosis (10-12).

Mcl-1 is unique among anti-apoptotic Bcl-2 homologues because it binds a subset of BH3 domain-only proteins that are different from those bound by Bcl-2 and Bcl-xL (13,14). In addition, Mcl-1 has been shown to play an apical role in the inhibition of apoptosis following UV irradiation (15). The ability of Mcl-1 to inhibit Bak in the mitochondria suggests that Bak is important for the anti-apoptotic of Mcl-1 (16). However, much less is known about the interaction between Mcl-1 and Bax. Recent studies have revealed that Mcl-1 inhibits the Bax-induced release of cytochrome c after the conformational change in Bax and its translocation into the mitochondria (17).

In the present study, we investigated the potential therapeutic effects of triptolide and its underlying mechanisms in human cervical cancer. We provide evidence that triptolide induces death in cervical cancer cells through the mitochondrial apoptotic pathway by inactivating Akt and decreasing Mcl-1 expression.

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Materials and methods

Cell culture and materials. HeLa and Caski cell lines derived from human cervical carcinomas were obtained from the American Type Culture Collection (Manassas, VA, USA). HeLa and Caski cells were cultured in DMEM medium (Gibco BRL, Gaithersburg, MD, USA) containing 10% fetal bovine serum (FBS) (HyClone, Logan, UT, USA) and 26 μM sodium bicarbonate for monolayer cell culture. Stock solutions of triptolide, z-VAD-fmk and LY294002 (Calbiochem, San Diego, CA, USA) were prepared in DMSO. Polyclonal anti-polyclonal anti-poly(ADP-ribose) polymerase (PARP), anti-Bc1-X_L and anti-actin antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA); anti-phospho Akt, anti-caspase-3 and anti-caspase-9 from Cell Signaling Technology (San Diego, CA, USA). Monoclonal antibodies against caspase-8 and Bcl-2 were purchased from Upstate Biotechnology (Santa Cruz, CA, USA) and Santa Cruz Biotechnology, respectively.

MTT assay. Cell viability was evaluated using the MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, a tetrazole] reduction conversion assay. Briefly, cells grown in 96-well plates were treated as required. Next, cells were incubated with 100 μl of MTT (5 mg/ml) for 2 h. The formazan crystals resulting from conversion of MTT by mitochondrial enzymatic activity were solubilized with Me_2SO, and absorbance was measured at 570 nm using a microplate reader (Molecular Devices, CA, USA). Cell survival was expressed as the absorbance of MTT-treated cells relative to that of untreated controls.

Reverse transcription-polymerase chain reaction (RT-PCR). Total RNA was extracted from HeLa cells using the TRIzol reagent (Invitrogen, Carlsbad, CA, USA). Complementary DNA was synthesized from 2 μg of total RNA using MMLV reverse transcriptase (Takara Co., Ltd. Japan). Human Mcl-1 mRNA was amplified using the sense primer 5'-CCT TCC AAG GAT GGG TTT GT-3' and the antisense primer 5'-TCT TCA ATC AAT GGG GAG CA-3'. The following PCR cycling conditions were used to amplify Mcl-1: 30 sec at 94˚C, 45 sec at 58˚C, 30 sec at 72˚C (30 cycles), and a 5-min extension at 72˚C. Reaction products were analyzed on a 1.0% agarose gel and visualized by ethidium bromide (EtBr).

Immunoblot analysis. Proteins were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and electrophoretically transferred to nitrocellulose membrane. The nitrocellulose membrane was blocked with 5% non-fat dry milk in phosphate-buffered saline (PBS)-Tween-20 (0.1%, v/w) at 4˚C overnight. The membrane was incubated with primary antibody (diluted according to the manufacturer's instructions) for 2 h. Horseradish peroxidase-conjugated anti-rabbit or anti-mouse IgG was used as the secondary antibody. Immunoreactive protein was visualized using enhanced chemiluminescence (Amersham, Arlington Heights, IL, USA). Signals on X-ray film were quantified with a scanning densitometer (Personal Densitometer, Molecular Dynamics, Sunnyvale, CA, USA) using area integration.

DNA fragmentation analysis and 4',6'-diamidino-2-phenylindole (DAPI) staining. Approximately 1x10^6 HeLa cells were lysed in buffer containing 10 mM Tris (pH 7.4), 150 mM NaCl, 5 mM EDTA, and 0.5% Triton X-100 for 30 min on ice. Lysates were vortexed and cleared by centrifugation at 12,000 x g for 30 min. Fragmented DNA was extracted from the supernatant with an equal volume of neutral phenol:chloroform:isoamyl alcohol mixture (25:24:1) and separated on a 1.5% agarose gel containing 0.1 μg/ml EtBr. HeLa cells were treated with triptolide for 24 h. The cells were fixed with 4% paraformaldehyde on a glass slide for 30 min at room temperature. Cells were then washed with PBS, incubated with 300 nM DAPI (Microprobe, CA, USA) for 10 min, and examined by fluorescence microscopy. Apoptotic cells were identified by condensation and fragmentation of nuclei. DAPI staining experiments were performed in duplicate.

Flow cytometric analysis of propidium iodide (PI) and JC-1 staining. Approximately 1x10^6 HeLa cells were suspended in 100 μl of PBS and supplemented with 200 μl 99% ethanol while being gently vortexed. The cells were incubated at 4˚C for 1 h, washed with PBS, and resuspended in 250 μl of 1.12% sodium citrate buffer and 12.5 μg of RNase. Cells were then incubated at 37˚C for 30 min. Cellular DNA was stained by incubating cells with 250 μl of PI (50 μg/ml) for 30 min at room temperature. The relative DNA content was then determined based on red fluorescence using fluorescence-activated cell sorting (FACS), which was performed using a FACScan flow cytometer. For the analysis of apoptosis, HeLa cells were stimulated with triptolide, and apoptosis was measured by flow cytometry following staining with PI. JC-1 is capable of selectively entering the mitochondria, where it forms monomers and emits green fluorescence (FL1) when the mitochondrial membrane potential ($\Delta\psi$m) is relatively low. At a high $\Delta\psi$m, JC-1 aggregates and emits red fluorescence (FL2). The excitation wavelength was set at 488 nm, and the emission of FL1 and FL2 was detected using 525-nm and 575-nm band pass filters, respectively.

Transfection of HeLa cells with Akt, Mcl-1 and Bcl-2 expression vectors. Plasmids encoding human Akt, Mcl-1, or Bcl-2 were a gift from Dr T.K. Kwon (Keimyung University, Korea). Transfections were performed using Lipofectamine Plus reagent (Invitrogen), according to the manufacturer's recommendations. Briefly, 1x10^5 HeLa cells were cultured in 6-well culture plates in 2 ml of DMEM supplemented with 10% FBS and incubated for 24 h. Cells were washed twice with Opti-MEM medium (Invitrogen) and incubated for 4 h in a mixture of 800 μl Opti-MEM medium and 200 μl Lipofectamine reagent containing 1 μg of expression vector (pcDNA 3.1 or Akt, Mcl-1, or Bcl-2 expressing vector). The medium was then replaced with 2 ml of DMEM supplemented with 10% FBS, and cells were incubated for an additional 24 h. HeLa cells were stably transfected with pcDNA 3.1-Bcl-2 plasmids, or control plasmid pcDNA 3.1 vector using Lipofectamine. After 48 h of incubation, transfected cells were selected in primary cell culture medium containing 700 μg/ml G418. To eliminate the possibility of clonal differences between generated stable cell lines, HeLa/pcDNA 3.1 and

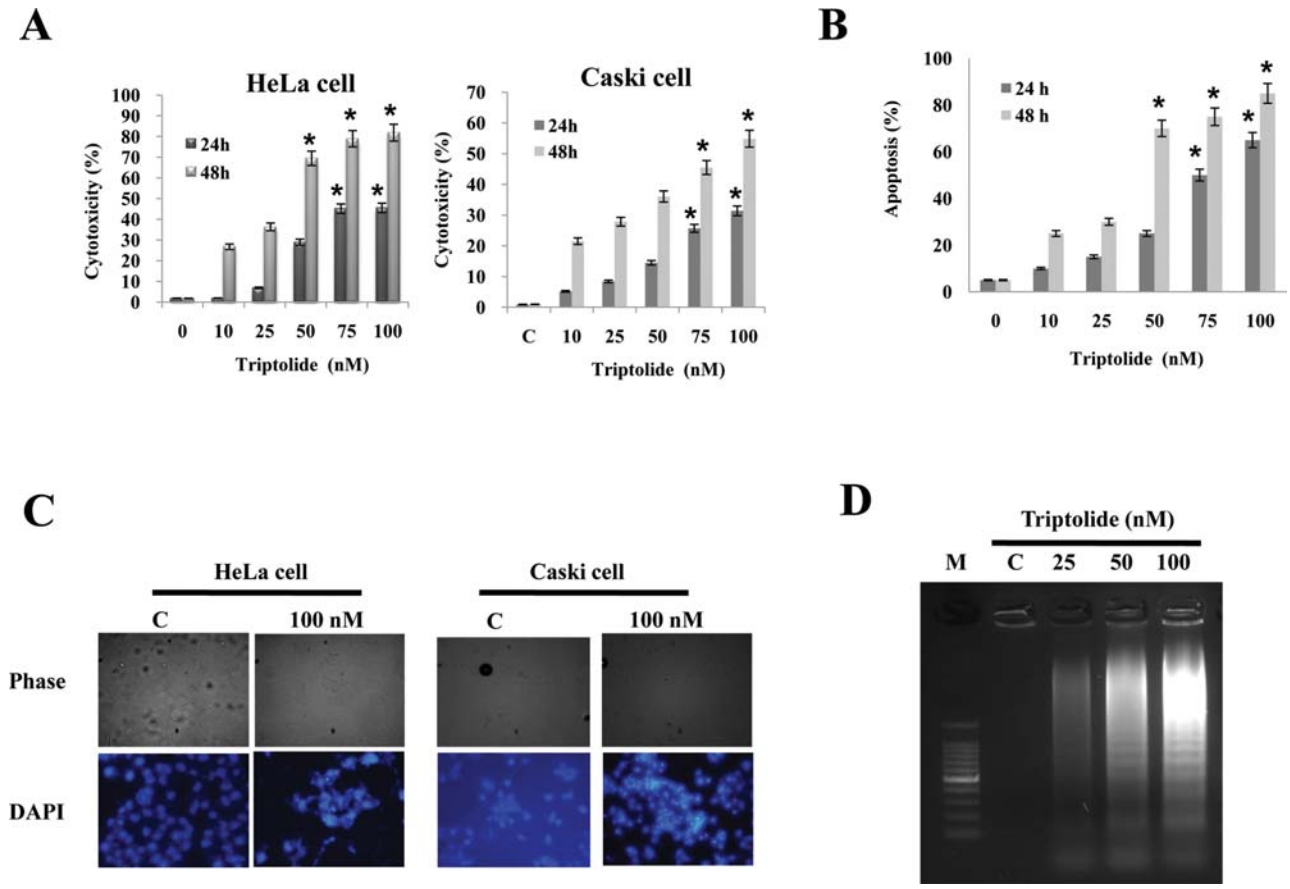

Figure 1. Cytotoxic effect of triptolide cervical cancer cells. (A) MTT assays showing viability of HeLa cells, Caski cells treated for 24 or 48 h with the indicated concentration of triptolide. Cell survival is expressed as the absorbance of MTT-treated cells relative to that of Me_2SO-treated controls. Data represent means ± SD (N=3). *P<0.05 compared to the control. (B) The percentage of apoptotic HeLa cells following treatment with the indicated concentrations of triptolide for 24 and 48 h. Apoptosis was assessed by DNA content after propidium iodide staining. Data are reported as the mean ± SD of three independent experiments. *P<0.05 compared to the control. (C) DAPI staining showing death of cervical cancer cells following exposure to 100 nM triptolide for 24 h. (D) Fragmentation of genomic DNA in HeLa cells treated for 24 h with the indicated concentrations of triptolide. Fragmented DNA was extracted and analyzed on a 2% agarose gel. C, control; M, marker.

HeLa/Bcl-2 clones were tested for Bcl-2 expression by immunoblotting after 3 or 4 weeks, and employed in subsequent experiments.

Analysis of cytochrome c release. Cells were harvested, washed once with ice-cold PBS, and gently lysed for 10 min in 60 μl of ice-cold lysis buffer (25 mM HEPES, pH 7.5; 250 mM sucrose; 1 mM EDTA; 1 mM EGTA; 1 mM DTT; 10 mM KCl; 1.5 mM $MgCl_2$; 1 mM PMSF; protease inhibitor cocktail). Lysates were centrifuged at 12,000 rpm for 20 min at 4˚C to obtain supernatants (cytosolic extracts free of mitochondria) and pellets (fraction that contains mitochondria). The resulting cytosolic fractions were analyzed by immunoblot using an anti-cytochrome c antibody.

Statistical analysis. Experiments were performed three or more separate times. Statistical analysis was performed using the Student t-test or analysis of variance. P<0.05 was considered significant.

Results

Induction of apoptosis by triptolide in cervical cancer cells. To verify that triptolide is cytotoxic, we analyzed the viability of human cervical cancer cells exposed to varying doses of triptolide. MTT assays revealed that treatment with triptolide for 24 and 48 h reduced the viability of HeLa and Caski cells in a concentration-dependent manner (Fig. 1A). To understand whether triptolide decreased cell viability by inducing apoptosis, we used two established criteria to assess apoptosis in our system, flow cytometric analysis of PI staining and analysis of DNA fragmentation. Flow cytometric analysis revealed that treatment of HeLa cells with triptolide markedly increased accumulation of sub-G1-phase cells in a concentration- and time-dependent manner (Fig. 1B). DNA fragmentation, another hallmark of apoptosis, was also increased by triptolide. In addition, triptolide markedly increased nuclear condensation and the formation of apoptotic bodies in both HeLa and Caski cells, as seen by DAPI staining (Fig. 1C). As shown in Fig. 1D, agarose gel electrophoresis of DNA from HeLa cells treated with triptolide for 24 h produced a typical ladder-like pattern of internucleosomal fragmentation. These results suggest that the cytotoxic effect of triptolide on cervical cancer cells is related to its apoptosis-inducing activity.

Effect of triptolide on caspase processing. Next, we investigated the possible molecular and cellular mechanisms

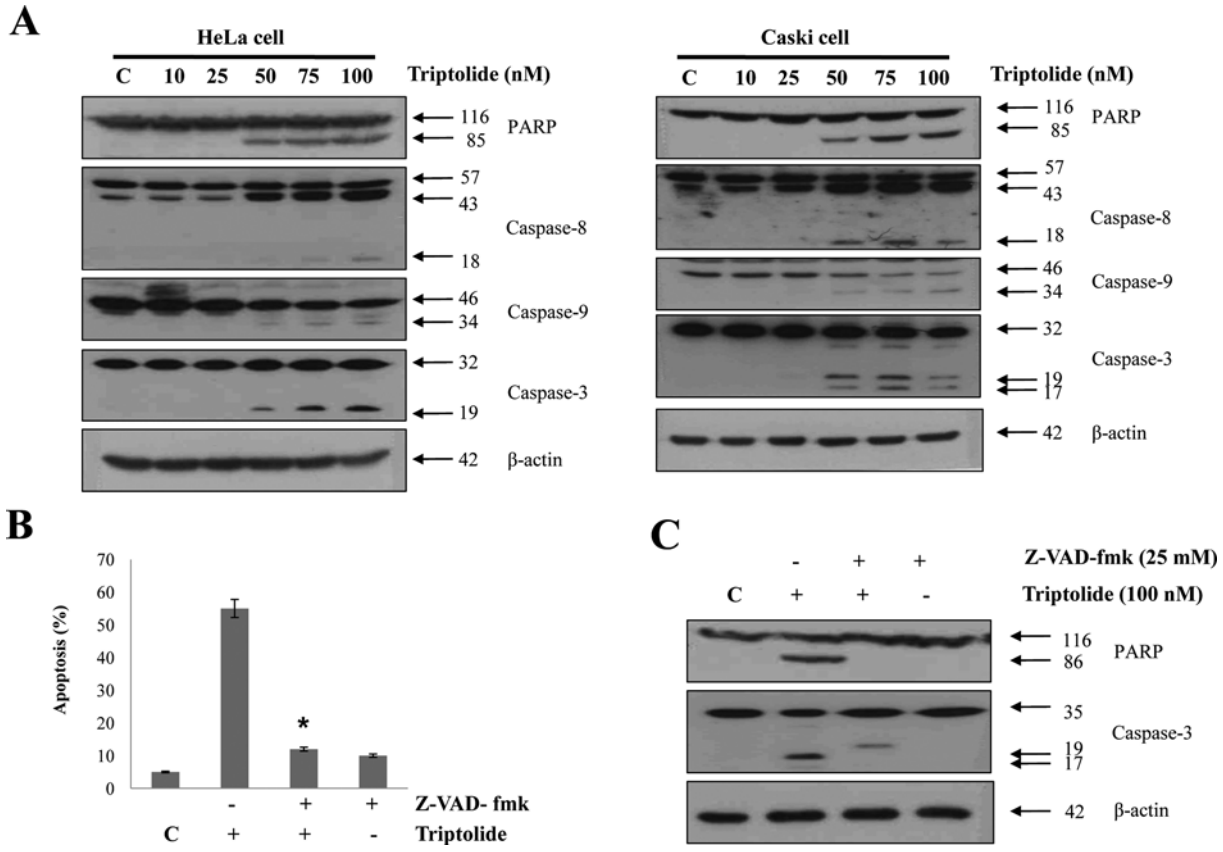

Figure 2. Effect of triptolide on caspase activation in cervical cancer cells. (A) Immunoblot analysis of caspase-8, caspase-9, caspase-3 and PARP in HeLa, Caski lysates (20 μg). Proteolytic fragments of the caspases and PARP are indicated with arrows. ß-actin served as a loading control. (B) Apoptosis in HeLa cells incubated with 25 μM z-VAD-fmk or solvent for 1 h before treatment with 100 nM triptolide for 1 h. Apoptosis was assessed by the DNA content after propidium iodide staining. Data are expressed as the mean (± standard deviation) obtained from three independent experiments. (C) Immunoblot analysis showing the effect of z-VAD-fmk on the cleavage of PARP and caspase-3. ß-actin served as a loading control.

underlying triptolide-induced apoptosis in cervical cancer cells. Because caspases participate in tumor cell death, we examined the effect of triptolide on the activation of caspases in cervical cancer cells. We found that both pro-caspase-8 and pro-caspase-9 were cleaved in HeLa and Caski cells treated with 50-100 nM of triptolide for 24 h. The same was true of caspase-3, which lies downstream of caspase-8 and caspase-9. The activation of caspases often leads to the proteolytic cleavage of target proteins, such as PARP. Immunoblot analysis showed that increasing concentrations of triptolide progressively increased the proteolytic cleavage of PARP in HeLa and Caski cells (Fig. 2A). To further evaluate caspase activation in triptolide-treated cells, we treated HeLa cells with a general, potent caspase inhibitor (z-VAD-fmk). As shown in Fig. 2B and C, triptolide-induced cell death was significantly suppressed when cells were pre-treated with z-VAD-fmk, indicating that triptolide-induced apoptosis is mediated by caspase-3 activation. These results clearly show that triptolide-induced apoptosis is associated with the activation of caspases.

Effect of triptolide on Bcl-2 family proteins in cervical cancer cells. Members of the Bcl-2 family, including Bcl-2 and Mcl-1, are anti-apoptotic proteins that favor the growth of cancer cells (18-20). In view of the critical role that Bcl-2 family proteins play in the regulation of apoptosis, we examined the expression of Bcl-2 proteins in cervical cancer cells treated with varying concentrations (0-100 nM) of triptolide for varying lengths of time (0-24 h). Treatment of HeLa cells with triptolide was not changed the expression of Bcl-2, Bcl-xL and Bax proteins (Fig. 3A). However, treatment of cervical cancer cells with the indicated concentration (10-100 nM) of triptolide at the indicated times (0-24 h) of triptolide markedly decreased levels of Mcl-1 (Fig. 3B). To determine whether triptolide decreased Mcl-1 protein levels through changes in transcription, we analyzed Mcl-1 mRNA levels using RT-PCR. Indeed, we found that Mcl-1 mRNA levels decreased in a manner dependent on the dose of triptolide (Fig. 3C). Actin mRNA levels were unaffected by triptolide treatment. These data strongly suggest that triptolide-induced downregulation of Mcl-1 expression is controlled at the transcriptional level.

Effect of triptolide on Akt phosphorylation in HeLa cells. The protein kinase, Akt, participates in cell proliferation, survival and transformation (21). Therefore, we investigated the effect of triptolide on Akt phosphorylation in HeLa cells. As shown in Fig. 4, HeLa cells expressed substantial levels of endogenous phosphorylated Akt, suggesting that Akt is constitutively activated in these cells. We observed a profound decrease in the amount of phosphorylated Akt in HeLa cells treated with >25 nM triptolide. Triptolide attenuated the

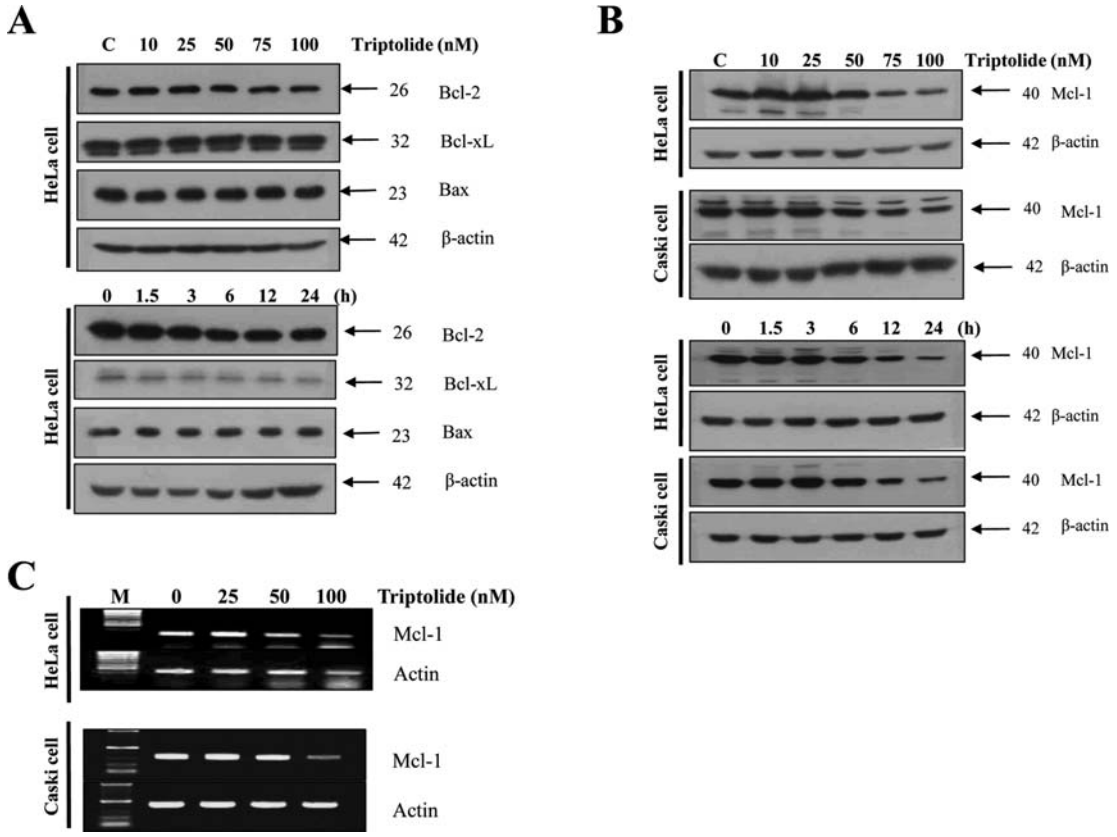

Figure 3. Effect of triptolide on Bcl-2 family proteins in cervical cancer cells. (A and B) Immunoblot analysis of Bcl-2, Bcl-xL and Bax (A) as well as Mcl-1 (B) in lysates (20 μg) from HeLa cells incubated with the indicated concentration of triptolide for 24 h or with 100 nM triptolide for the indicated time. ß-actin served as a loading control. (C) RT-PCR analysis of Mcl-1 mRNA levels in HeLa and Caski cells incubated for 24 h with the indicated concentrations of triptolide. ß-actin mRNA served as the control.

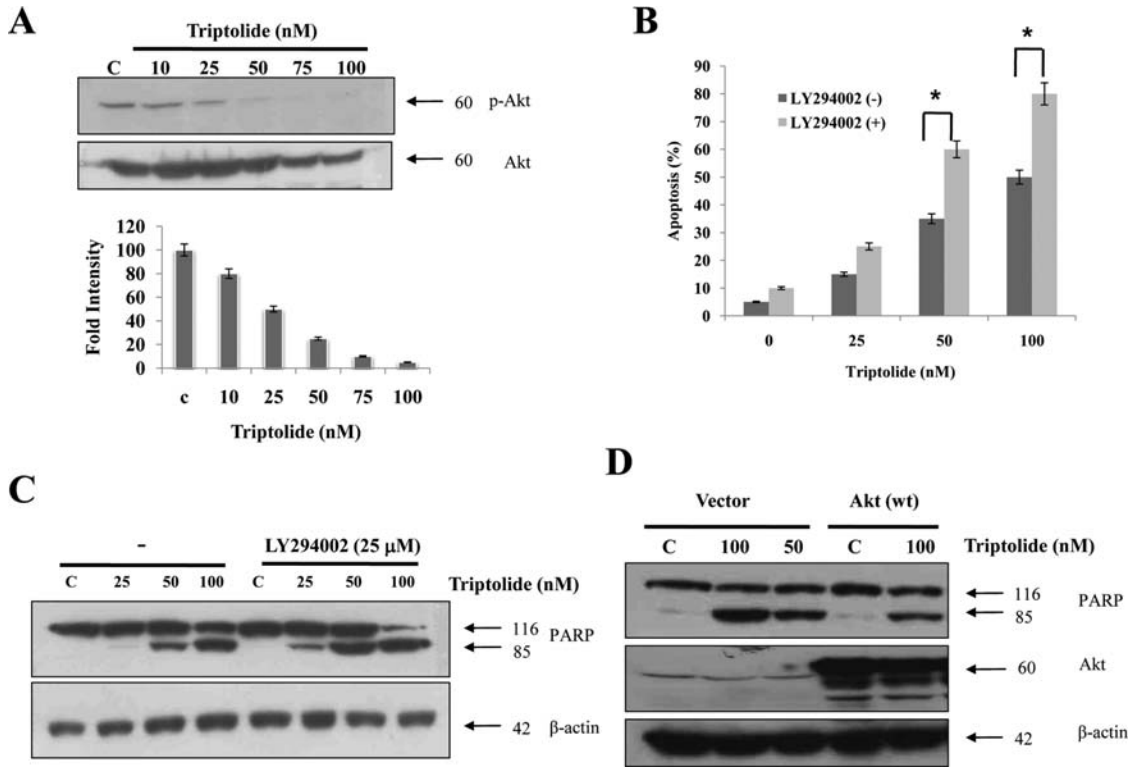

Figure 4. Effect of triptolide on PI3K/Akt signaling in HeLa cells. (A) Immunoblot analysis of phospho-Akt and Akt in HeLa cells exposed to the indicated concentrations of triptolide for 24 h. In the bar graph, the Y-axis is the intensity of p-Akt/Akt. (B) Apoptosis in HeLa cells treated for 24 h with the indicated concentrations of triptolide with or without 25 μM LY294002. Apoptosis was assessed by DNA content after propidium iodide staining. *P<0.05 compared to LY294002(-) cells. (C) Immunoblot analysis showing the effect of LY294002 on PARP cleavage. ß-actin served as a loading control. (D) Immunoblot analysis of PARP Akt and ß-actin in HeLa cells transfected with empty vector or Akt (wt) and then treated with or without triptolide for 24 h.

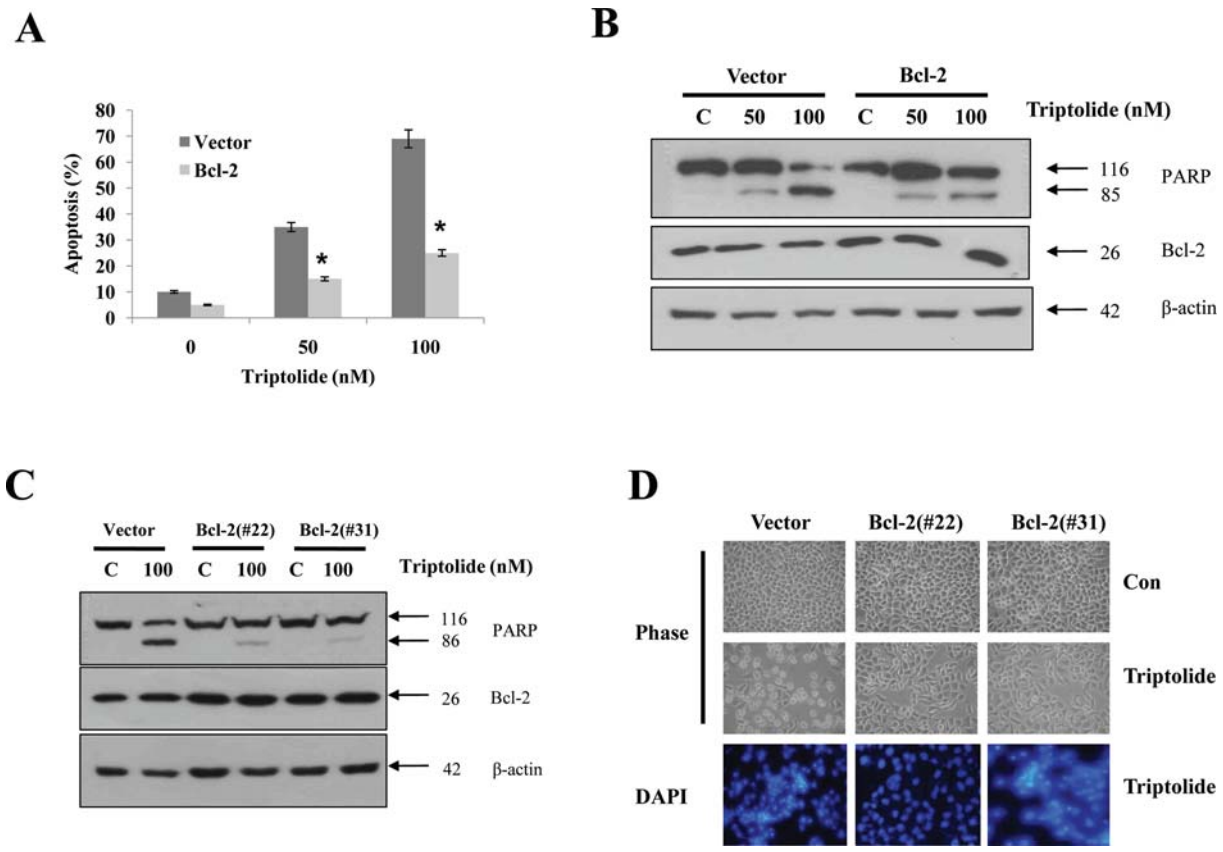

Figure 5. Effect of Bcl-2 overexpression on triptolide-induced death in cervical cancer cells. (A) Apoptosis in HeLa cells transfected with pcDNA (HeLa/vector) or Bcl-2 (HeLa/Bcl-2) and then treated with the indicated concentrations of triptolide for 24 h. Apoptosis was assessed by DNA content after propidium iodide staining. Data represent means ± SD (N=3). *P<0.05 versus vector. (B) Immunoblot analysis of Bcl-2, PARP and ß-actin in HeLa/vector and HeLa/Bcl-2 cells exposed to the indicated concentrations of triptolide for 24 h. (C) Immunoblot analysis of Bcl-2, PARP and ß-actin in cell lines that stably expressed Bcl-2 (HeLa/Bcl-2 no. 22 and HeLa/Bcl-2 no. 31). Cells were exposed to 100 nM triptolide for 24 h. (D) DAPI staining showing resistance of HeLa/Bcl-2 no. 22 and HeLa/Bcl-2 no. 31 cells to death following exposure to 100 nM triptolide for 24 h.

ability to phosphorylate Akt in HeLa cells. Importantly, PI staining (Fig. 4B) and immunoblot analysis of PARP cleavage (Fig. 4C) showed that triptolide-mediated apoptosis was exacerbated by LY294002, an inhibitor of the Akt-activating kinase, PI3K. This suggests that triptolide induce apoptosis by negatively regulating Akt. This idea was tested by analyzing the effect of Akt overexpression on triptolide-induced apoptosis in HeLa cells. We found that apoptosis, as assessed by PARP cleavage, occurred less frequently in HeLa cells expressing ectopic Akt than in vector-transfected HeLa cells (HeLa/vector cells). Together, these results suggest that downregulation of PI3K/Akt signaling plays an important role in triptolide-induced apoptosis in cervical cancer cells.

Effect of Bcl-2 overexpression on triptolide-induced death in HeLa cells. To investigate the role of Bcl-2 in triptolide-mediated apoptosis, we analyzed triptolide cytotoxicity (50 or 100 nM triptolide for 24 h) in HeLa cells overexpressing Bcl-2 (HeLa/Bcl-2 cells). FACS analysis of PI staining revealed that triptolide treatment markedly decreased the accumulation of sub-G1 phase cells in HeLa/Bcl-2 cells but not in HeLa/vector cells (Fig. 5A). Triptolide also increased cell death, as seen by a lower level of PARP cleavage in HeLa/Bcl-2 cells than in HeLa/vector cells (Fig. 5B). To further quantify apoptosis induced by triptolide, we analyzed PARP cleavage (Fig. 5C) as well as nuclear condensation and fragmentation (Fig. 5D) in cell lines stably overexpressing Bcl-2 (HeLa/Bcl-2 no. 22 and HeLa/Bcl-2 no. 31). We found that HeLa/vector cells treated with 100 nM triptolide exhibited nuclear condensation and the formation of apoptotic bodies, as seen by DAPI staining. In contrast, chromatin was rarely condensed and fragmented in triptolide-treated HeLa/Bcl-2 no. 22 cells or HeLa/Bcl-2 no. 31 cells.

Triptolide-induced activation of the mitochondrial cell death pathway in HeLa cells. To understand which of the apoptotic signaling pathways is essential for triptolide-induced caspase-dependent cell death, we explored whether triptolide-induced cell death requires caspase-9, which is activated through the mitochondrial signaling pathway for cell death. To do this, we transiently transfected HeLa cells with plasmid encoding dominant-negative caspase-9 (caspase-9 dN) and treated these cells with triptolide for 24 h. As shown in Fig. 6A, caspase-9 dN cells, but not vector-transfected cells, were resistant to triptolide, suggesting that caspase-9 is essential for triptolide-induced cell death and that triptolide induces apoptosis via the mitochondrial pathway. To further show that triptolide-induced cell death occurs via the mitochondrial pathway, we measured cytochrome c release and $\Delta\psi m$ in

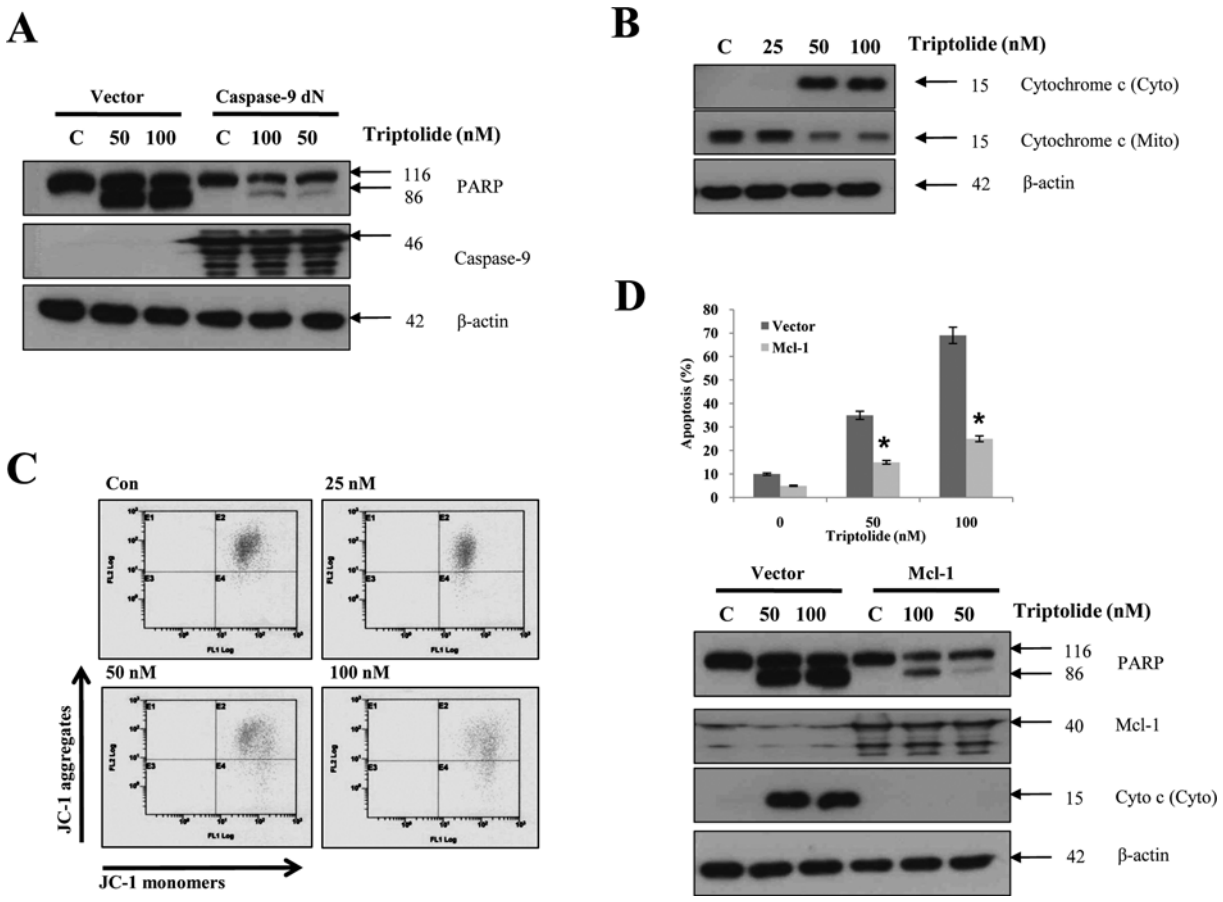

Figure 6. Induction of mitochondrial apoptotic events by triptolide in HeLa cells. (A) Immunoblot analysis of caspase-9 in HeLa cells that were transfected with pcDNA (vector) or caspase-9 dN and treated with or without triptolide for 24 h. (B) Immunoblot analysis of cytochrome c in cells treated with triptolide for 24 h. (C) Flow cytometric analysis of $\Delta\psi m$ in cells treated with the indicated concentration of triptolide. $\Delta\psi m$ was determined with JC-1. (D) HeLa cells that were transfected with pcDNA (HeLa/vector) or Mcl-1 (HeLa/Mcl-1) were treated with or without triptolide for 24 h. PARP, Mcl-1, cytochrome c and β-actin levels were determined by immunoblot, and apoptosis was determined by DNA content (PI staining). Data represent means ± SD (N=3). *P<0.05 versus vector.

HeLa cells. We were unable to detect cytochrome c levels in the cytosol of untreated cells; however, cytochrome c was greatly increased in the cytosol of cells treated with triptolide (50 or 100 nM) (Fig. 6B). Furthermore, $\Delta\psi m$ was substantially decreased in cells exposed to triptolide (50 or 100 nM) (Fig. 6C). Finally, we hypothesized that if triptolide-induced cell death is indeed mediated through inactivation of Mcl-1, then Mcl-1 levels will determine the extent to which triptolide induces cell death. To test this idea, we investigated the effect of Mcl-1 overexpression on triptolide-induced death of HeLa cells. As expected, triptolide induced considerable death in HeLa/vector cells (Fig. 6D), as assessed by PI staining, PARP cleavage and cytochrome c release. However, cell death was attenuated by Mcl-1 overexpression. Together, our findings support the idea that triptolide induces apoptosis through the mitochondrial pathway and that elimination of Mcl-1 by triptolide promotes mitochondrial injury.

Discussion

Many efforts have been made in the therapeutic application of triptolide in chemoprevention. In our study, triptolide induced apoptosis in HeLa and Caski cervical cancer cells. Our data suggest that the apoptotic effect of triptolide on cervical cancer cells may result from the modulation of activities and/or the expression of multiple cellular factors, including caspases, Bcl-2, Mcl-1 and Akt. Our investigation of the underlying apoptotic signaling pathway in cervical cancer cells yielded two important findings. First, we found that triptolide-induced death of cervical cancer cells was dependent on Akt inactivation and caspase-9 activation. Secondly, we identified Mcl-1 as an anti-apoptotic factor upstream of the mitochondria that plays a central role in countering the apoptosis pathway initiated by triptolide.

The PI3K/Akt pathway is a well-characterized signaling pathway that blocks apoptosis in a variety of cell types (22). Interestingly, overexpression of PI3K/Akt is observed in cancer cells, and increased PI3K/Akt signaling in cancer cells is thought to impart resistance to anti-cancer drugs (23). In this study, we found that HeLa cells contained substantial levels of phosphorylated Akt (Fig. 4A). Therefore, sustained Akt activity in these cells may facilitate their growth and/or survival. Triptolide has been shown to enhance PS-341 (bortezomib, proteasome inhibitor)-induced apoptosis via the PI3K/Akt/nuclear factor-κB pathway in dexamethasone-resistant human multiple myeloma cells (24). Moreover, triptolide leads to the inactivation of the PI3K/Akt pathway in drug-resistant chronic myelogenous leukemia cells (25). However, we found that triptolide had an inhibitory effect on Akt in HeLa cells (Fig. 4). Because Akt is a pro-survival

protein, the inhibition of Akt may be involved in triptolide-mediated growth suppression of HeLa cells.

A variety of cellular proteins participate in the induction of apoptosis. Among these are the caspases, which are essential for the execution of cell death induced by apoptotic stimuli (26). We have demonstrated that triptolide exposure leads to the processing of caspase-8, -9 and -3 in cervical cancer cells (Fig. 2). An important finding was that triptolide induced caspase-3 processing and PARP cleavage in parallel with the induction of apoptosis. Furthermore, the inhibition of caspase-3 by the broad-spectrum caspase inhibitor z-VAD-fmk blocked cell death by triptolide, suggesting that triptolide-induced cell death is caspase-dependent (Fig. 2B and C). Triptolide has been reported to induce caspase-dependent apoptosis in leukemia cells by degrading XIAP (4). However, XIAP protein was less affected by triptolide in cervical cancer cells.

The finding that transfection of caspase-9 dN imparted resistance to triptolide indicates that the activation of caspase-9 and subsequent activation of caspase-3 activation (i.e., the mitochondrial pathway of caspase activation) are critical to the action of triptolide (Fig. 6A). The release of mitochondrial intermembrane space proteins to the cytosol is a key event during apoptosis (27,28). Release of cytochrome c is required for the initiation of the apoptosome formation and the activation of caspases, while Smac/DIABLO and Omi/HtrA2 are believed to enhance caspase activation through the neutralization of the inhibitors of apoptosis proteins. Our results clearly showed that triptolide induced cytochrome c release into the cytosol (Fig. 6B) and decreased $\Delta\psi m$ (Fig. 6C), supporting the idea that triptolide activates the mitochondrial apoptotic pathway.

Members of the Bcl-2 family, including Bcl-2 and Mcl-1, negatively regulate apoptosis and caspase activation by modulating mitochondrial membrane integrity (18-20). We observed that triptolide-induced apoptosis occurred through the inhibition of Mcl-1, with this event being followed by a loss of $\Delta\psi m$ and activation of the cytochrome c/caspase-9 pathway. These observations are consistent with previous studies showing that triptolide induces cytochrome c release, which causes apoptosis through activation of caspases (4). Here, triptolide effectively inhibited Mcl-1 expression at both the protein and mRNA levels (Fig. 3B and C, respectively). Several studies have implicated E2F1, CREB and ETS transcription factors in the control of Mcl-1 gene expression (29-31). Our finding that triptolide substantially decreased Mcl-1 mRNA levels suggests that triptolide downregulates Mcl-1 expression by repressing promoter activity or destabilizing mRNA.

Here, we found that forced expression of Mcl-1 markedly diminished triptolide-mediated death in HeLa cancer cells. This finding has important therapeutic implications, as it shows that the downregulation of Mcl-1 plays a critical role in triptolide-induced lethality. In accord with our finding, Mcl-1 overexpression also largely inhibited PARP cleavage and the release of cytochrome c into the cytosol. These findings are consistent with the studies described by Nijhawan and colleagues (15), who demonstrated that Mcl-1 operates upstream of Bax and Bcl-xL translocation to the mitochondria, cytochrome c release and caspase activation in UV-treated HeLa cells. Interestingly, in our study, a decrease in Mcl-1 protein alone was not sufficient to induce apoptosis, suggesting that one or more additional perturbations are necessary for triptolide-induced cell death. Another study showed that Mcl-1 interacts with tBid and impairs the ability of tBid to induce the release of cytochrome c and apoptosis (32). Recently, two separate groups used BH3 peptides to show that BH3-only proteins can differentially target anti-apoptotic Bcl-2 proteins (13,14).

In summary, triptolide induces apoptosis in cervical cancer cells, likely by inducing multiple cellular events such as caspase activation, Mcl-1 inactivation, inhibition of Akt signaling, and loss of $\Delta\psi m$. The findings presented here suggest that triptolide is a potential anti-cancer drug for cervical cancer.

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