Triptolide induces apoptosis in human adrenal cancer NCI-H295 cells through a mitochondrial-dependent pathway

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Abstract. Triptolide, the main active component obtained from *Tripterygium wilfordii* Hook. f, has been reported to present potent immunosuppressive and anti-inflammatory biological activities. It has been previously shown that due to the cytotoxicity of triptolide it has a limited use in the clinic. Although numerous studies have shown that triptolide induced apoptosis in many human cancer cells there is no report to show triptolide-induced apoptosis in human adrenal cancer cells. We treated the human adrenal cancer NCI-H295 cells with triptolide *in vitro* and investigated its cytotoxic effects. The cytotoxicity was examined and quantitated by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) assay and the viability of inhibition and apoptosis was determined by flow cytometric assay, using propidium iodide (PI) staining for apoptosis. Flow cytometric assay also used for the determination of reactive oxygen species (ROS) production and the levels of mitochondrial membrane potential ($\Delta\Psi_m$), and the caspase-3 and -9 activation in NCI-H295 cells. Western blotting was used for examining the changes of apoptotic associated proteins. The results indicated that triptolide induced cytotoxicity (decreased the percentage of viable cells) and induced sub-G1 phase (apoptosis) occurring in NCI-H295 cells and those effects are dose-dependent. Results also showed that triptolide promoted the production of ROS and decreased the levels of $\Delta\Psi_m$ in examined NCI-H295 cells. The results showed that triptolide promoted the levels of cytochrome c, Apaf-1, AIF, Endo G, caspase-9 and -3 which were analyzed by Western blotting. These results suggest that triptolide is able to induce apoptosis on NCI-H295 cells through the mitochondrial-dependent signal pathway.

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Introduction

Triptolide, is a diterpenoid triepoxide, which is isolated from the plant *Tripterygium wilfordii* Hook. f (a member of the Celastraceae family) (1) that has been use in Chinese traditional medicine for two centuries. In patients, triptolide has been reported to treat a variety of autoimmune diseases, including rheumatoid arthritis; nephritis; and systemic lupus erythematosus and as immunosuppressant (2-5).

Besides the anti-inflammatory and immunosuppressive activities, triptolide has been shown to have antitumor properties in a variety of human tumor cells via impairing cell growth and inducing apoptosis (1,6-8). However, a subacute toxicological study from mice demonstrated that the kidney is one of the target organs for triptolide (9). However, the effect of triptolide on human adrenal cancer NCI-H295 cells has not yet been investigated. Thus, we tried to identify the mechanism of triptolide-induced apoptosis in human adrenal cancer cells *in vitro*.

The present study was focused to define, for the first time, the anticancer effects of triptolide in treating human adrenal cancer NCI-H295 cells. We investigated whether triptolide was able to mediate the inhibition of cell growth and induction of apoptosis in human adrenal cancer NCI-H295 cells. Furthermore, to identify a potential signal pathway for the anticancer effect in triptolide treated adrenal cancer cells. Our results demonstrated that triptolide induced NCI-H295 cells death in apoptotic manner through a mitochondrial-dependent pathway. Furthermore, caspase-3 and -9 activations are also observed during the course of apoptosis in NCI-H295 cells.

Materials and methods

Materials and chemicals. Triptolide, potassium phosphate, dimethyl sulfoxide (DMSO), propidium iodide, triton X-100, Tris-HCl, trypan blue, ribonuclease-A were purchased from Sigma Chemical Co. (St. Louis, MO, USA). RPMI-1640 medium, L-glutamine, fetal bovine serum, penicillin-streptomycin, and trypsin-EDTA were obtained from Invitrogen Corp. (Carlsbad, CA, USA). The primary antibodies were obtained as follows: antibodies for caspase-9 and caspase-3 were purchased from Cell Signaling Technology (Beverly, MA); antibodies for cytochrome c, Apaf-1, ß-actin, AIF, Endo G and horseradish peroxidase (HRP)-linked goat anti-mouse IgG, goat anti-rabbit IgG, were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Caspase-9 inhibitor (Z-LEHD-FMK) and caspase-3 inhibitor (Z-DEVD-FMK) were obtained from R&D Systems (Minneapolis, MN, USA) then were dissolved in DMSO and diluted in cell culture medium before use.

Cell culture. The human adrenal cancer NCI-H295 cells were obtained from the Food Industry Research and Development Institute (Hsinchu, Taiwan). Cells were cultured in 75 cm^2 tissue culture flasks at 37˚C under a humidified 5% CO_2 atmosphere, with 90% RPMI-1640 medium with 2 mM L-glutamine adjusted to contain 10% fetal bovine serum (Grand Island, NY), and 1% penicillin-streptomycin (100 U/ml penicillin and 100 μg/ml streptomycin).

Examination of morphological changes and determination of cell viability and sub-G1 phase of cell cycle. Approximately $2x10^5$ cells/well of NCI-H295 cells were grown in 12-well plates for 24 h before they were treated with triptolide at 0, 50, 100, 150 and 200 μM and cells were incubated at 37˚C, 5% CO_2 and 95% air for 48 h. For morphological changes, cells were examined and photographed under phase-contrast microscope as described previously (10,11). For cell viability assay by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay, cells ($1x10^4$ cells/well) were plated in 96-well plates and exposed to triptolide for 48 h, MTT (0.5 mg/ml) was added to each well and cells were incubated for an additional 4 h at 37˚C. The medium was aspirated from the wells and the blue formazon product was dissolved in 100 μl of DMSO. The plates were read at OD 570 nm using a spectrophotometric plate reader (Bio-Rad, Tokyo, Japan) as previously described (12,13). Each data point was replicated in triplicate. For sub-G1 (apoptosis) determinations, the harvested cells were fixed gently in 70% ethanol at 4˚C overnight and then re-suspended in PBS containing 40 μg/ml PI and 0.1 mg/ml RNase and 0.1% Triton X-100 in the dark for 30 min at 37˚C. The cells were analyzed with a flow cytometer (Becton-Dickinson, San Jose, CA, USA) equipped with an argon ion laser at 488-nm wavelength (14).

Caspase-9 and -3 activities and caspase inhibitors inhibit triptolide-induced cell death in NCI-H295 cells. NCI-H295 cells ($5x10^5$ cells/well) were pretreated with caspase-3 inhibitor (Z-DEVD-FMK), a caspase-9 inhibitor (Z-LEHD-FMK) and a pan-caspase inhibitor (Z-VAD-FMK) before cells were treated with 125 μM of triptolide then were incubated at 37˚C, 5% CO_2 and 95% air for 48 h. NCI-H295 cells were harvested for determination of cell viability as described above. Cells were harvested by centrifugation and the cell pellets were added in lysis buffer (50 mM Tris-HCl, 1 mM EDTA, 10 mM EGTA, 10 mM digitonin and 2 mM DTT) on ice for 10 min. Cell lysates (50 μg proteins) after centrifugation at 15,000 x g at 4˚C for 10 min were incubated with caspase-3 and -9 specific substrates (Ac-DEVD-pNA and Ac-LEHD-pNA; R&D Systems) with reaction buffer in a 96-well plate at 37˚C for 1 h. The caspase activity was determined by measuring OD 405 of the released pNA as describe elsewhere (15).

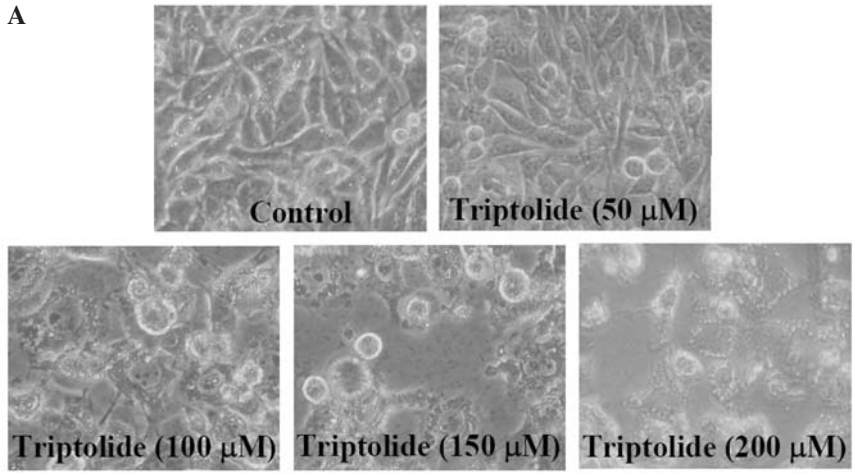
Reactive oxygen species (ROS) and mitochondrial membrane potential ($\Delta\Psi_m$) determinations. NCI-H295 cells at a density of $2x10^5$ cells/well were plated onto 12-well plates and treated with 100 μM of triptolide for 0, 6, 12, 18 and 24 h. All cell samples were harvested then re-suspended in 500 μl of DCFH-DA (10 μM) for ROS (H_2O_2) determination, suspended in 500 μl of DiOC6 (1 μmol/l) for $\Delta\Psi_m$ determination and then incubated at 37˚C for 30 min and analyzed by flow cytometry (16,17). All fluorescence intensities were obtained from the mean intensity of the histogram constructed from 10,000 cells.

Western blotting of apoptosis associated proteins. NCI-H295 cells at a density of $1x10^7$ cells/well were treated with 125 μM of triptolide for 0, 6, 12 and 24 h. Cells from each treatment were collected by centrifugation and the total proteins were quantitated (16,17). Equal amounts of lysate protein were run on 12% SDS-PAGE and electrophoretically transferred to PVDF membrane (Amersham Pharmacia Biotech, USA) then the blocked blots were incubated with specific primary antibody against cytochrome *c*, Apaf-1, AIF, Endo G, caspase-9 and caspase-3 overnight and further incubated for 1 h with HRP conjugated secondary antibody (Santa Cruz Biotechnology). Bound antibodies were detected by ECL kit as described previously (18-21).

Statistical analysis. Student's t-test was used to analyze differences between treated and control groups; $p<0.05$, $p<0.01$, $p<0.001$.

Results

Triptolide induces morphological changes and decreases the percentage of viable human adrenal cancer NCI-H295 cells. To investigate the morphological changes and inhibition of cell viability of NCI-H295 cells after exposure to triptolide, the cells were assessed for their potential cytotoxicity on human adrenal cancer cells *in vitro*. Control cells appeared in the well spreading in spindle shape after observed with the phase contrast microscope (Fig. 1A). After treatment with 50, 100, 150 and 200 μM of triptolide for 24 h, the cell shape changed to round (Fig. 1A), the round-shaped cells suggest that they were undergoing apoptosis initiated by triptolide. The results in Fig. 1 indicate that triptolide induced a significant increase in cell viability inhibition.



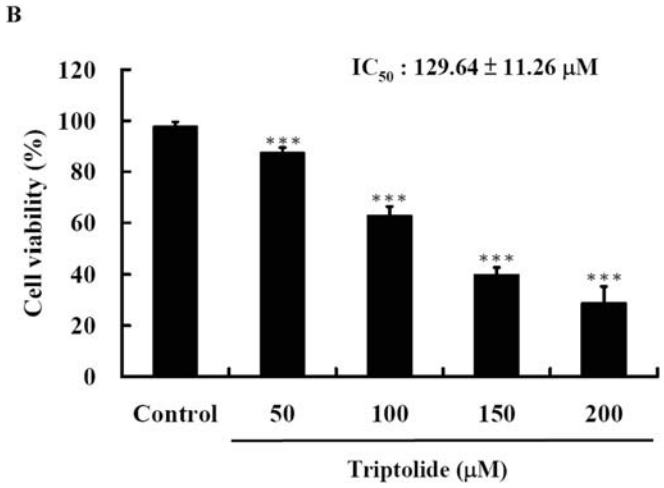


Figure 1. Triptolide induces morphological changes and decreases the percentage of viable human adrenal cancer NCI-H295 cells. NCI-H295 cells ($2x10^5$ cells/well) were grown in a 12-well plate for 24 h then treated with 0, 50, 100, 150 and 200 μM of triptolide for 48 h. The cells were examined and photographed under contrast-phase microscope for morphological changes (A) and the total viable cells were determined (B) as described in Materials and methods. Each point is mean ± SD of three experiments. ***P<0.001 indicates a significant difference from the control.

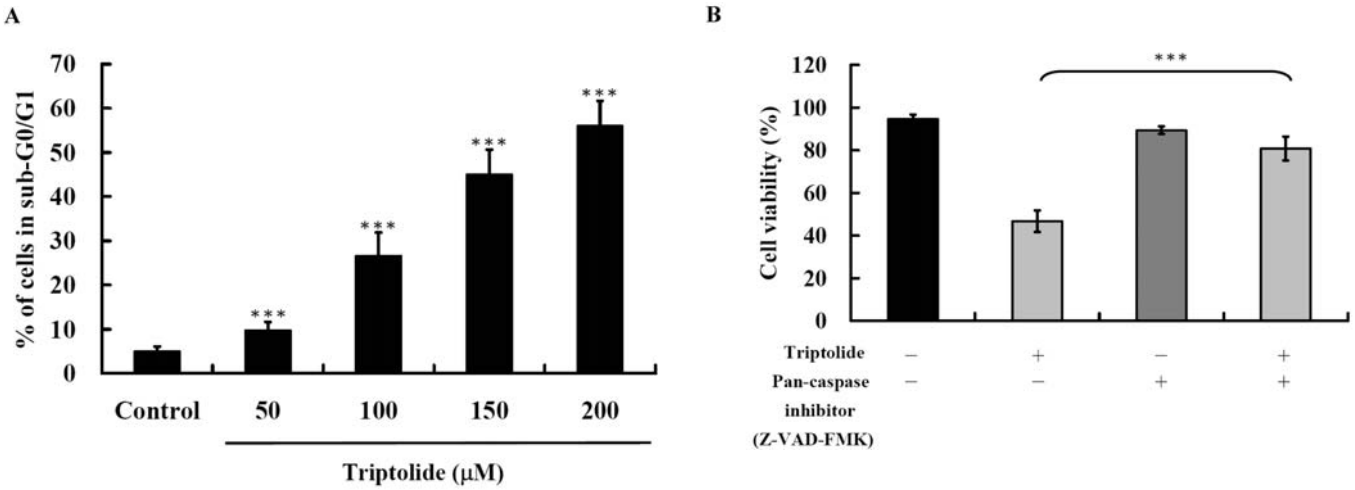


Figure 2. Triptolide induces apoptosis (sub-G1) and caspase inhibitors affect the viability of NCI-H295 cells *in vitro*. NCI-H295 cells ($5x10^5$ cells/well) were pre-treated with pan-caspase inhibitor (Z-VAD-FMK) and then were treated with 0, 50, 100, 150 and 200 μM of triptolide for 48 h. Then cells were harvested for assaying the sub-G1 group of cell cycle (A) and the percentage of viable cells (B) were counted by flow cytometric assay as described in Materials and methods. The percentage of apoptosis was calculated. Each point is the mean ± SD of three experiments. ***P<0.001 indicates a significant difference from the control.

Triptolide induces apoptosis (sub-G1) and caspase inhibitors affect the viability of NCI-H295 cells in vitro. It is well documented that cells undergoing early apoptosis can be seen in the sub-G1 phase of the cell cycle. NCI-H295 cells after exposure to various doses of triptolide were harvested and analyzed by flow cytometry and results are shown in Fig. 2A, indicating that triptolide induced apoptosis on NCI-H295 cells in a dose-dependent manner. NCI-H295 cells were pretreated with pan-caspase inhibitors then with 125 μM of triptolide and analyzed for the percentage of viable cells. The results

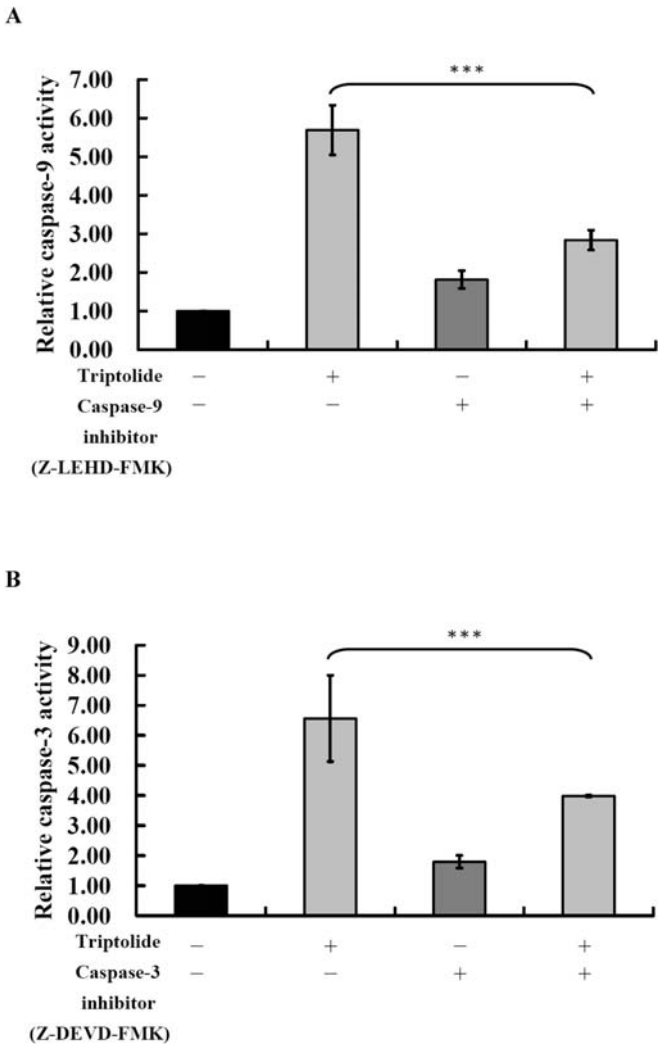


Figure 3. Caspase-9 and -3 inhibitors inhibited triptolide-induced caspase-9 and -3 activities in NCI-H295 cells. NCI-H295 cells ($5x10^5$ cells/well) were pretreated with caspase-3 inhibitor (Z-DEVD-FMK), caspase-9 inhibitor (Z-LEHD-FMK) and pan-caspase inhibitor (Z-VAD-FMK) before cells were treated with 125 μM of triptolide then incubated at 37˚C, 5% CO_2 and 95% air for 48 h. Cells were harvested by centrifugation and the cell pellets were add to 50 μl of 10 μM caspase-3 and -9 substrate solution. The samples were incubated at 37˚C for 60 min before flow cytometric analysis. Caspase-3 and -9 activities were detectable in the FL-1 channel (a BD instrument with emission at 525 nm). ***P<0.001 indicates a significant difference from the control.

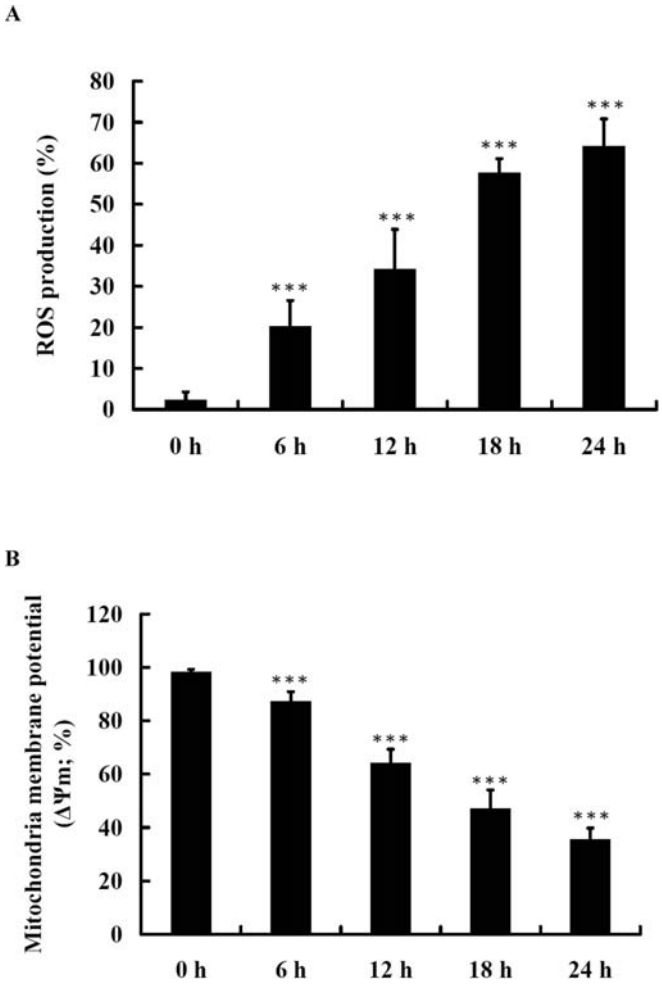


Figure 4. Triptolide affects the production of ROS and the levels of $\Delta\Psi_m$ in NCI-H295 cells. NCI-H295 cells ($2x10^5$ cells/ml) were treated with 125 μM of triptolide for 0, 6, 12, 18 and 24 h. The cells were harvested and re-suspended in 500 μl of 2,7-Dichlorodihydrofluorescein diacetate (DCFH-DA) (10 μM) for ROS and in 500 μl of DiOC6 (1 μmol/l) for $\Delta\Psi_m$. Cells was incubated at 37˚C for 30 min then were analyzed by flow cytometry. ***p<0.001 indicates a significant difference from the control.

are shown in Fig. 2B, indicating that triptolide decreased the viable cells, however, the cells pretreated with pan-caspase inhibitors then exposed to triptolide showed an increase in the percentage of viable cells compared to triptolide treatment only.

Caspase-9 and -3 inhibitors inhibite triptolide-induced caspase-9 and -3 activities in NCI-H295 cells. Caspase-9 and -3 play important roles in agent induced apoptosis through a mitochondrial-dependent pathway, furthermore, caspase-3 is a key executioner of apoptosis, and is responsible for the proteolytic cleavage of many other key proteins such as PARP-1. NC-H295 cells were treated with 125 μM triptolide for 48 h then analyzed by flow cytometric assay and results are shown in Fig. 3A and B indicating that triptolide activated caspase -9 and -3 in a dose-dependent manner. However, the cells pretreated with inhibitors of caspase-9 and -3 and exposed to triptolide showed a decrease in the activation of caspase-9 and -3.

Triptolide affects the production of ROS and the levels of $\Delta\Psi_m$ in NCI-H295 cells. It is well documented that ROS and mitochondria play an important role in agent-induced apoptosis; therefore, we investigated whether triptolide promoted ROS production and increased the levels of $\Delta\Psi_m$ in NCI-H295 cells. NCI-H295 cells were treated with 125 μM triptolide for 48 h then analyzed by flow cytometric assay and the results are shown in Fig. 4A and B. Triptolide activated caspase -9 and -3 in a dose-dependent manner. However, when cells were pretreated with inhibitors of caspase-9 and -3 and then exposed to triptolide this led to a decrease in the activation of caspase-9 and -3.

Triptolide affects the levels of apoptosis-associated proteins in NCI-H295 cells. In order to investigate and confirm the

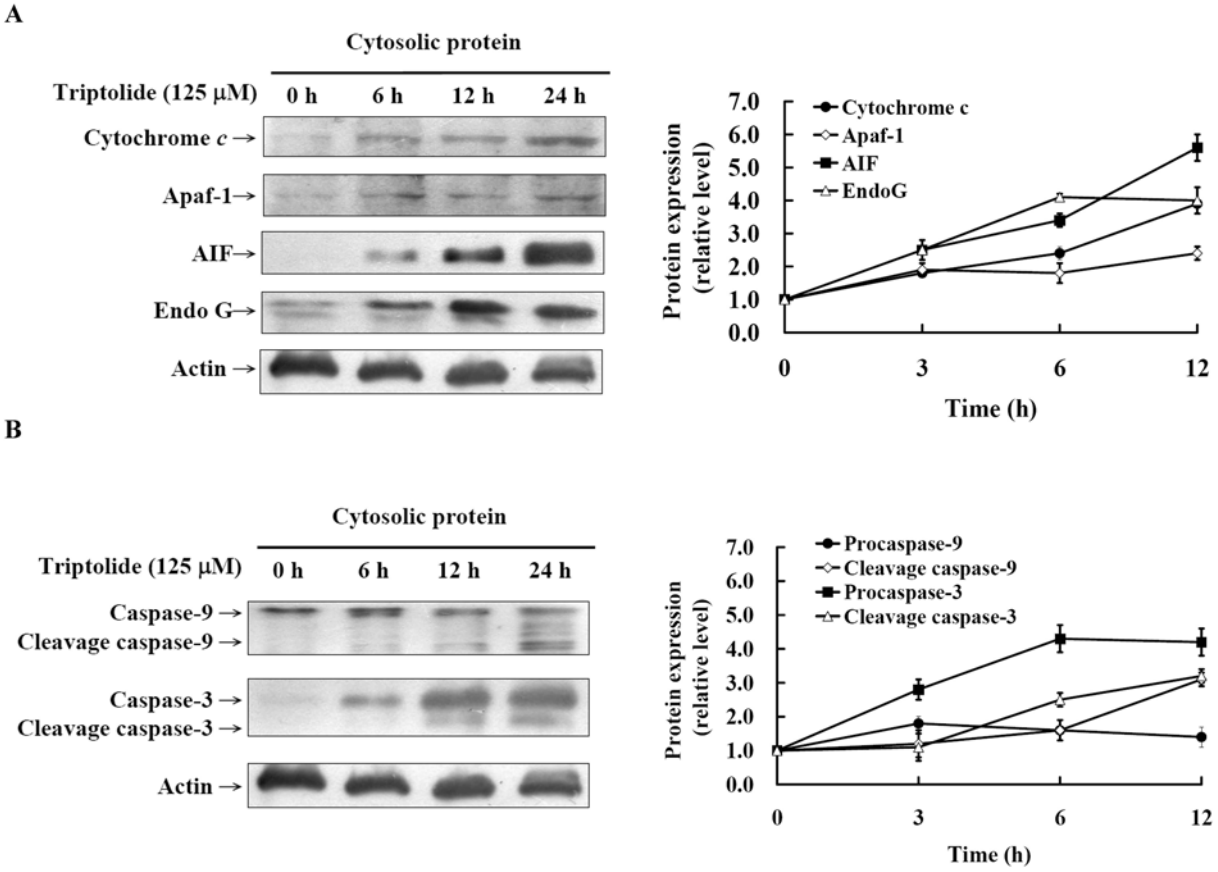


Figure 5. Triptolide affects the protein levels of apoptosis in NCI-H295 cells. The NCI-H295 cells were treated with 125 μM triptolide for 0, 6, 12 and 24 h and the total proteins were prepared, then detected by Western blotting. The primary antibodies for cytochrome c, Apaf-1, AIF and Endo G (A), caspase-9 and caspase-3 were used (B) then each sample was stained by secondary antibody as described in Materials and methods.

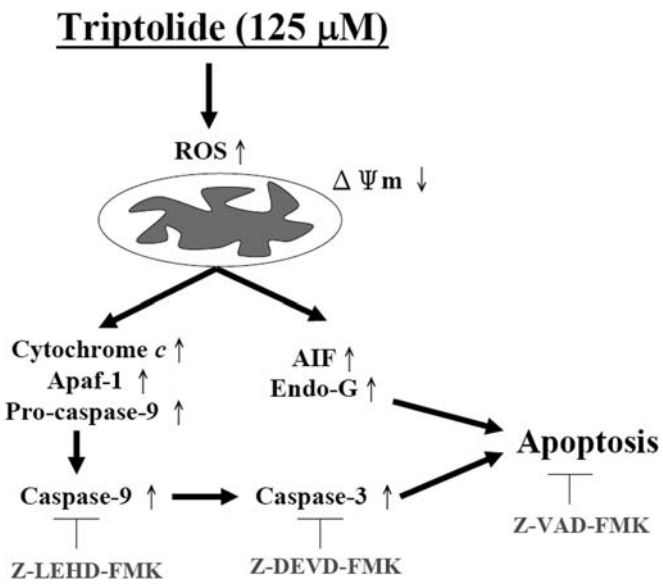


Figure 6. The proposed signaling pathways of triptolide-induced apoptosis in human adrenal cancer NCI-H295 cells are presented.

molecular signaling pathways of triptolide induced apoptosis in NCI-H295 cells, the cells were treated with triptolide and total proteins were collected. Western blotting was used for examining the changes of apoptosis associated proteins and results are shown Fig. 5A and B. The results indicated that triptolide promoted the levels of cytochrome c, Apaf-1, AIF and Endo G (Fig. 5A) and activated caspase-9 and -3 (Fig. 5B) which also indicated that triptolide decrease the level of $\Delta\Psi_m$ then led to cytochrome c, AIF and Endo G release from mitochondria.

Discussion

Numerous studies have shown that triptolide has anti-inflammatory effects (22,23), contraceptive activity (24,25), and anti-neoplastic abilities (26,27). However, due to it severe toxic effects the clinical use of triptolide is limited. Recently, several investigators demonstrated that triptolide promote apoptosis in a variety of cell types (28-33) including normal human proximal tubular epithelial HK-2 cells *in vitro* (34). In this study, we report that triptolide induced apoptosis in human adrenal cancer NCI-H295 cells. Triptolide induced significant viability inhibition and morphological changes of cells and induced apoptosis in triptolide-treated NCI-H295 cells. Moreover, triptolide promoted ROS production and dysfunction of mitochondria. Finally, we found triptolide was able to activate caspase-9 and -3, thus providing a reasonable explanation for its induction of apoptosis in NCI-H295 cells. These results suggest that triptolide could be usefully investigated as a possible therapeutic agent for adrenal cancer cells.

Whether the cytotoxicity (decreased the percentage of viable cells) caused by triptolide on NCI-H295 cells is due to apoptosis or necrosis could not be based on the number of viable cells. To clarify this point, we also used Western blotting for examining the changes of apoptotic associated proteins and caspase-9 and -3 activities in triptolide-treated NCI-H295 cells.

Apoptosis is a tightly regulated autonomously programmed cell death which is involved and utilized extensively during the development and maintenance of tissue and organ homeostasis (35) and elimination of damaged cells. Numerous evidence indicates that many anticancer drugs can cause the death of tumor cells through induction of apoptosis. It is well-known that apoptosis can be divided into two pathways such as the death receptor-mediated extrinsic pathway and the mitochondria-mediated intrinsic pathway (36,37). Our results showed that triptolide induced apoptosis through mitochondria-mediated intrinsic pathway based on several observations such as i) decreased levels of $\Delta\Psi_m$; ii) cytochrome c, AIF and Endo G release from mitochondria and iii) activation of caspase-9 and -3 in examined NCI-H295 cells.

It was also reported that mitochondrion plays a critical role in apoptosis induced by some drugs (38). The mitochondrial death pathway is involved in the changes in the permeability of the outer mitochondrial membrane with the collapse of membrane potential (39,40). After the dysfunction of mitochondria cytochrome c was released and in some signals may also cause AIF and Endo G release from mitochondria. Our results from Western blotting also showed that triptolide increased the release of cytochrome c, AIF and Endo G in NCI-H295 cells. It was reported that the cytosolic cytochrome c from mitochondria binds to Apaf-1, leading to the activation of caspase-9, caspase-3 and poly (ADP-ribose) polymerase (41,42). Caspase-3 plays an important role in the apoptotic pathways and is expressed in almost all types of cells as inactive pro-enzyme (43,44). Caspase-3 can be activated by other activated caspases such as caspase-8 and -9, and subsequently cleaves some specific substrate, i.e. PARP (45) and the cleavage of DNA repair enzyme PARP resulted in chromosomal DNA break and finally led to apoptosis (46). Our results also clearly showed that triptolide induced similar results. Therefore, we suggest that triptolide induced apoptosis through the mitochondria-dependent pathway.

In conclusion, we demonstrated that triptolide significantly induces apoptosis in NCI-H295 cells and the possible signal pathways are shown in Fig. 6. This apoptotic response is associated with the loss of mitochondrial membrane potential, cytochrome c, AIF and Endo G release from mitochondria and activation of caspase-9 and -3. Therefore, we believe that triptolide might be a promising molecule in cancer chemoprevention or chemotherapy in adrenal cancer; and further efforts to explore this therapeutic strategy are necessary.

Acknowledgements

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