

Cantharidin induces G2/M phase arrest by inhibition of Cdc25c and Cyclin A and triggers apoptosis through reactive oxygen species and the mitochondria-dependent pathways of A375.S2 human melanoma cells

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Abstract. Cantharidin (CTD), a component of natural mylabris (*Mylabris phalerata* Pallas) was reported to have high cytotoxicity in many human cancer cell lines. However, it was not reported to affect human melanoma A375.S2 cells. In the present study, we found that CTD induced cell morphological changes and decreased the percentage of viable cells and induced G2/M phase arrest and induction of apoptosis in A375.S2 cells. Results also showed that CTD induced the generation of reactive oxygen species (ROS) and Ca²⁺ and decreased mitochondria membrane potential and lead to the release of cytochrome *c*, AIF and Endo G. Further investigation revealed that CTD induced A375.S2 cells with an increase of caspase activation and caspase-dependent apoptotic proteins to trigger correlated pathway mechanisms according to western blotting results. Western blotting was used for examining the changes of G2/M phase arrest and apoptosis-associated protein expression and confocal laser microscopy was used to examine the translocation apoptosis-associated protein.

Results showed that CTD increased the protein expression of caspase-3, -8 and -9, cytochrome *c*, Bax, Bid, Endo G and AIF but inhibited the levels of Bcl-2 and Bcl-x. CTD induced ER stress-associated protein expression such as GRP78, IRE1 β , ATF6 α and caspase-12. Based on those observations, we suggest that CTD may have potential as a novel anti-cancer agent for the treatment of skin cancer.

Introduction

Human melanoma, an aggressive skin cancer, accounts for 10% of all skin cancers, but was estimated to be involved in >80% of deaths from skin cancers (1). In Western countries, skin cancer melanoma is becoming more common and resulting in increased mortality (2). In the USA, the incidence of melanoma has increased by 15-fold in the last 40 years (1,3). In individuals of European origin, the incidence of melanoma is still rising (4). Survival ratio for metastatic melanoma is low and the 10-year survival rate for patients with metastatic melanoma is <10% (5,6). It was reported that human melanoma is highly resistant to conventional chemotherapy (7). Currently, the effective treatment of human melanoma such as surgery, radiation, chemotherapy or a combination of radiotherapy with chemotherapy is not satisfactory. Thus, numerous studies had focused on finding novel potent drugs from natural products to combat this disease.

In nature, insects produce different defensive molecules against predators, and these molecules may be clinically used as medicinal drugs for therapeutic purposes (8). The dried body of mylabris (*Mylabris phalerata* Pallas) has been used in Chinese traditional medicine for the treatment of cancer (9). Cantharidin (CTD), a terpenoid, was isolated from mylabris (blister beetles) and other insects and was shown to induce cancer cell apoptosis in leukemia (10), myeloma (11), bladder (12), breast (13), colon (14), liver (15), pancreatic (16) and lung (17). It was reported that CTD inhibits migration and

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invasion of A549 human lung cancer cells via the inhibition of matrix metalloproteinase 2 (18). Recently, we also found that CTD induces cell apoptosis through mitochondria-dependent pathways (18) and induced DNA damage and inhibits DNA repair-associated protein levels in NCI-H460 human lung cancer cells (19).

Numerous studies have shown that CTD induced cytotoxic effects in many human cancer cell lines through the induction of apoptosis, however, there is no available information to show CTD-induced apoptosis in human skin cancer cells. Therefore, in the present study, A375.S2 human melanoma cells were selected for use as a cell model to investigate the anti-melanoma potential of CTD *in vitro*. The results indicated that CTD induced G2/M phase arrest and cell apoptosis in A375.S2 cells via the caspase- and mitochondrial-dependent signaling pathways.

Materials and methods

Chemicals and reagents. CTD, propidium iodide (PI), Trypsin-EDTA, dimethyl sulfoxide (DMSO) and DAPI were purchased from Sigma Chemical Co. (St. Louis, MO, USA). CTD was dissolved in DMSO to make a stock solution. Minimum essential medium (MEM), fetal bovine serum (FBS), L-glutamine and penicillin-streptomycin were purchased from Gibco®/Invitrogen Life Technologies (Carlsbad, CA, USA). Primary antibody such as WEE1, Cdc25c, Cyclin A, CDK1, p21, Fas, Fas-L, AIF, Endo G, cytochrome *c*, caspase-3, -8 and -9, Bax, Bid, Bcl-2, Bcl-x, XBP-1, GADD153, GRP78, caspase-12, IRE1 β , ATF6 α and Calpain 1 and peroxidase conjugated secondary antibodies were purchased from Cell Signaling Technology, Inc. (Beverly, MA, USA). The enhanced chemiluminescence (ECL) detection system was obtained from Amersham Life Science, Inc. (Arlington Heights, IL, USA).

Cell culture. The A375.S2 human malignant melanoma cancer cells were obtained from the Food Industry Research and Development Institute (Hsinchu, Taiwan). The cells were cultured in MEM supplemented with 10% FBS, 1% antibiotics (100 U/ml penicillin and 100 μ g/ml streptomycin) and 2 mM L-glutamine (Gibco®/Invitrogen Life Technologies, Grand Island, NY, USA) and maintained at 37°C with 5% CO₂ in a humidified atmosphere. The medium was changed every 2 days (20–22).

Observation of morphological changes and measurement of viable cells. A375.S2 cells (2x10⁵ cells/well) were seeded into 12-well plates for 24 h. CTD diluted in DMSO was then individually added to a final concentration of 0, 1, 2, 3, 4 and 5 μ M, and an equal amount of DMSO was added to the well as the control group for 48 h. The cellular morphology was observed and photographed by using a phase contrast microscope at magnification of x200. Cells from each well were harvested for the measurement of percentage of viability using a flow cytometric method (BD Bioscience FACSCalibur flow cytometer; Becton-Dickinson, San Jose, CA, USA) as described previously (20).

Measurement of cell cycle distribution by flow cytometry. A375.S2 cells (2x10⁵ cells/well) were seeded into 12-well

culture plates for 24 h and then were incubated with 0, 1, 2, 3, 4 and 5 μ M of CTD, or only with vehicle (DMSO, 1% in culture media) for 24 and 48 h. Cells were harvested by centrifugation and washed with phosphate-buffered saline (PBS). Then cells were fixed with 70% ethanol overnight at least for 24 h at 4°C and were washed twice with PBS and stained with 1 ml PI working solution (100 μ g/ml RNase A, 40 μ g/ml PI and 0.1% Triton X-100) for cellular staining at room temperature for 30 min in the dark. Analysis of cell cycle distribution was performed by a flow cytometer and analyzed by Cell Quest software package (BD Bioscience FACSCalibur flow cytometer; Becton-Dickinson) as described previously (20). Each experiment was repeated three times.

Reactive oxygen species (ROS), intracellular Ca²⁺ and mitochondrial membrane potential ($\Delta\Psi_m$) assays. Flow cytometry was used for measuring the levels of ROS, Ca²⁺ and $\Delta\Psi_m$ in A375.S2 cells. Briefly, A375.S2 cells (2x10⁵ cells/well) placed in 12-well plates for 24 h were then treated with 4 μ M of CTD for various time periods. The cells were collected from each timer point and then re-suspended in 500 μ l of DCFH-DA (10 μ M) for 30 min for ROS (H₂O₂) measurement, re-suspended in 500 μ l of DiOC₆ (4 μ M) for 30 min for the levels of $\Delta\Psi_m$ measurement and re-suspended in 500 μ l of Fluo-3/AM (2.5 μ g/ml) for 30 min for intracellular Ca²⁺ measurement and all samples were analyzed by flow cytometry as described previously.

Caspase-3, -8 and -9 activity assay. A375.S2 cells (2x10⁵ cells/well) were seeded onto 12-well plates for 24 h and then were pre-treated with Z-VAD-FMK, Z-IETD-FMK, Z-LEHD-FMK and Z-DEVD-FMK (inhibitors of caspase-pan, -8, -9 and -3, respectively) and then treated with 4 μ M of CTD for 0, 6, 24 and 48 h. Then cells were harvested and washed with PBS, and were re-suspended in 50 μ l of 10 μ M substrate solution of caspase-8, -9 and -3 substrates (CaspasLux8-L1D2, CaspasLux9-M1D2 and PhiPhiLux-G1D2, respectively) (OncoImmunin, Inc., Gaithersburg, MD, USA) for 30 min in the dark. Cells were measured for the activities of caspase-8, -9 and -3 by using flow cytometric assay as described previously (20).

Western blotting. A375.S2 cells (1x10⁶ cells/dish) were placed in 10 cm dish for 24 h and then were incubated with or without 4 μ M CTD for 0, 6, 12, 24 and 48 h then lysed in an ice-cold lysis buffer [10 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1 mM EGTA, 0.3 mM PMSF, 0.2 mM sodium orthovanadate, 0.1% SDS, 1 mM EDTA, 1% NP-40, 10 mg/ml leupeptin, and 10 mg/ml aprotinin], followed by denaturation. Then centrifuged at 13,000 rpm for 20 min at 4°C, before getting the supernatant to measure protein concentration by using a Bio-Rad protein assay kit (Bio-Rad, Hercules, CA, USA). Protein (30 μ g) was electrophoresed in 12% SDS-PAGE gel at 4°C, steady flow (10 mA in composition gel, 15 mA in separation gel) followed by transfer onto nitrocellulose membranes. The membranes were blocked with 5% skim milk in TBST (20 mmol/l Tris-HCl at pH 8.0, 150 mmol/l NaCl, and 0.05% Tween-20) for 1 h at room temperature, and then probed with relevant primary antibodies (anti-WEE1, Cdc25c, Cyclin A, CDK1, p21, Fas, Fas-L, AIF, Endo G, cytochrome *c*,

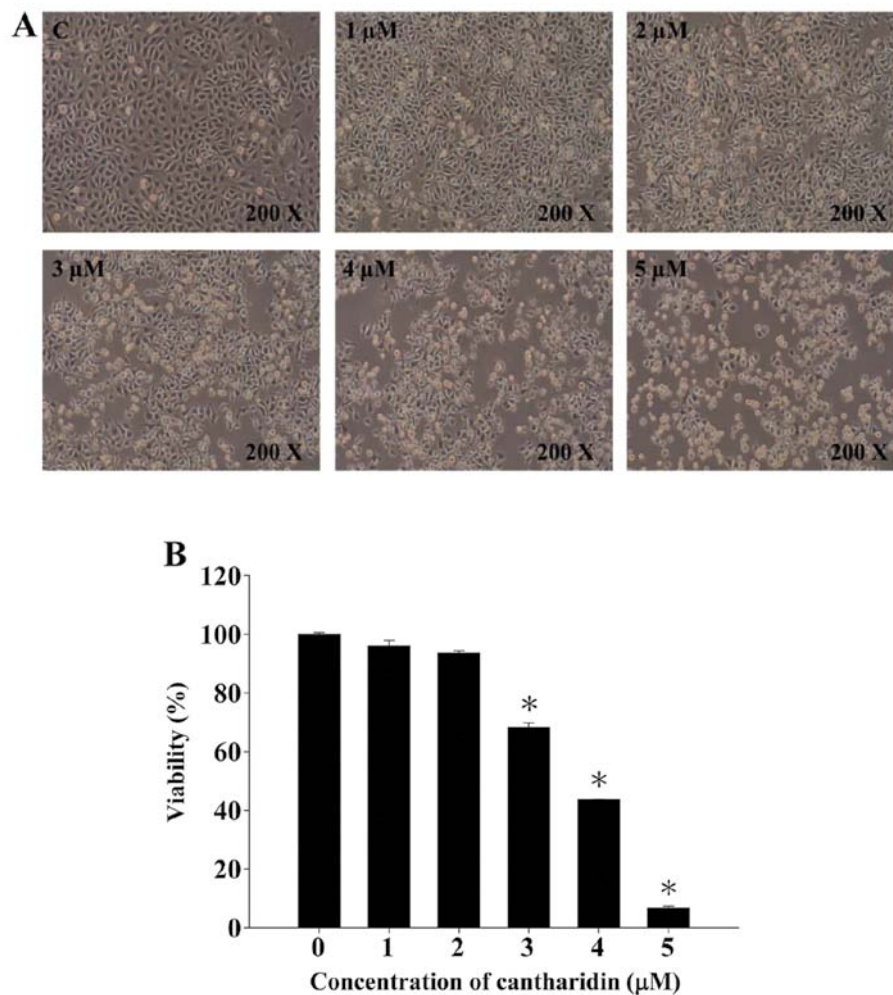


Figure 1. Cantharidin (CTD) induced cell morphological changes and decreased the percentage of viable A375.S2 cells. A375.S2 cells (2×10^5 cells/well) were treated with 0, 1, 2, 3, 4 and 5 μ M of CTD for 48 h before (A) the cells were photographed for examining the cell morphological changes and (B) were harvested for the percentage of viable cell measurements and analyzed by flow cytometry as described in Materials and methods. * $P < 0.05$, significant difference between CTD-treated groups and the control as analyzed by Student's t-test.

caspase-3, -8 and -9, Bax, Bid, Bcl-2, Bcl-x, XBP-1, GADD153, GRP78, caspase-12, IRE1 β , ATF6 α and Calpain 1) overnight at 4°C followed by peroxidase-conjugated secondary antibody for 1 h at 25°C. Proteins on the membrane were visualized by ECL detection (Amersham Biosciences ECL™) and exposed to X-ray film and bands obtained were quantified using NIH Image analyzer (NIH, Bethesda, MD, USA). β -actin staining served as the internal standard for the membranes. All of the western blots were performed at least three times (21,22).

Confocal laser scanning microscopy assay. A375.S2 cells (5×10^4 cells/well) were placed on 4-well chamber slides and incubated with or without 4 μ M CTD for 48 h and then fixed in 4% formaldehyde in PBS for 15 min, and they were permeabilized using 0.3% Triton X-100 in PBS for 1 h, followed using 2% BSA for blocking non-specific binding sites. Cells were stained by primary antibodies such as anti-Endo G, anti-cytochrome *c* and anti-AIF (all in green fluorescence) overnight and then washed with PBS. Cells were incubated with fluorescein isothiocyanate-conjugated second antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA) followed by mitotracker (red fluorescence) staining for

nuclein examination. The stained cells were analyzed with Leica TCS SP2 Confocal Spectral Microscope as described previously (23,24).

Statistical analysis. All data were expressed as mean \pm SD from triplicate experiments. Statistically significant differences between the CTD-treated and -untreated (control) groups were assessed by Student's t-test with SPSS 11.0 statistic software. $P < 0.05$ was considered statistically significant.

Results

CTD induced cell morphological changes and decreased the cell viability of A375.S2 cells. A375.S2 cells were pre-incubated with 0, 1, 2, 3, 4 and 5 μ M of CTD for 48 h then cells were photographed by phase contrast microscopy and were harvested for the total percentage of viable cells and the results are shown in Fig. 1A and B. Fig. 1A shows that CTD induced cell morphological changes. Fig. 1B shows a significant dose-dependent reduction of living cells with CTD treatment when compared to the control groups in A375.S2 cells and these effects are dose-dependent.

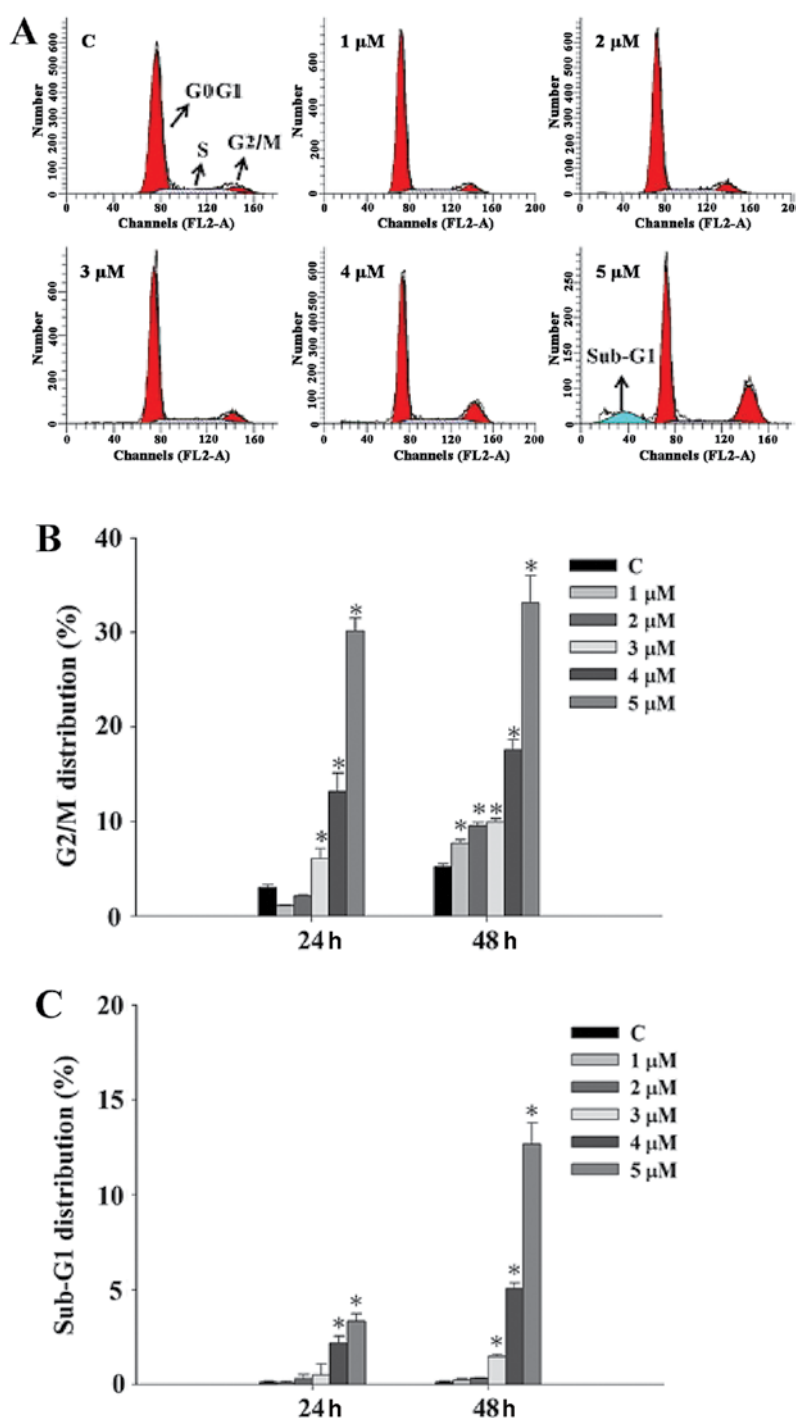


Figure 2. Cantharidin (CTD) induced sub-G1 phase and G2/M phase arrest of A375.S2 cells. A375.S2 cells were treated with 0, 1, 2, 3, 4 and 5 μ M of CTD for 24 and 48 h before the cells were examined for cell cycle distribution and sub-G1 phase of cell cycle [(A) 48 h profiles; (B) percentage of G2/M phase; (C) percentage of sub-G1 phase] and analyzed by flow cytometry as described in Materials and methods. * $P < 0.05$, significant difference between CTD-treated groups and the control as analyzed by Student's t-test.

CTD induces G2/M phase arrest and sub-G1 phase (apoptosis) of A375.S2 cells. A375.S2 cells were treated with various doses of CTD for 24 and 48 h before the cells were examined for sub-G1 phase in cell cycle assay by flow cytometry and the results are shown in Fig. 2A-C. Fig. 2A shows representative profiles from flow cytometry assay indicating that CTD induced sub-G1 phase and G2/M phase arrest in A375.S2 cells. Data in Fig. 2B and C indicate that CTD induced G2/M phase arrest and induced sub-G1 phase development, respectively,

and these effects are dose-dependent. At the 48-h treatment of CTD, a higher percentage of G2/M phase arrest and sub-G1 phase (apoptosis) was recorded than that of control groups.

CTD induces ROS and Ca^{2+} production and decreases the levels of $\Delta\Psi_m$ in A375.S2 cells. In order to confirm whether CTD induced apoptotic cell death in A375.S2 cells via the production of ROS and Ca^{2+} or dysfunction of mitochondria, cells were treated with CTD then analyzed by flow cytometry

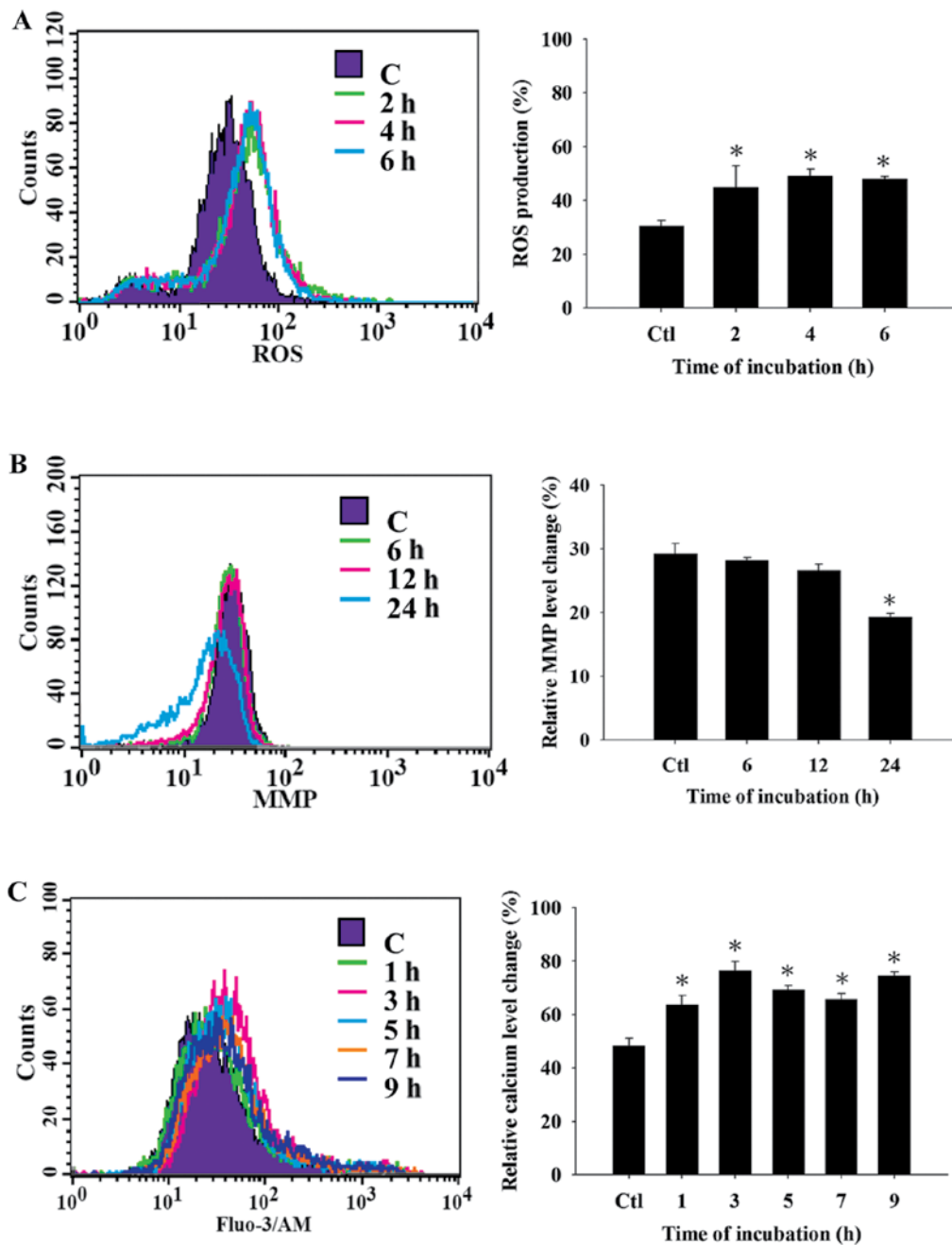


Figure 3. Cantharidin (CTD) induces reactive oxygen species (ROS) and Ca^{2+} production and decreased the levels of mitochondrial membrane potential ($\Delta\Psi_m$) in A375.S2 cells. A375.S2 cells (2×10^5 cells/well) were treated with $4 \mu\text{M}$ of CTD for various time periods. (A) Cells were isolated and suspended in $500 \mu\text{l}$ of DCFH-DA ($10 \mu\text{M}$) for ROS (H_2O_2), (B) re-suspended in $500 \mu\text{l}$ of DiOC₆ ($4 \mu\text{mol/l}$) for the levels of $\Delta\Psi_m$ measurement and (C) re-suspended in $500 \mu\text{l}$ of Fluo-3/AM ($2.5 \mu\text{g/ml}$) for intracellular Ca^{2+} concentrations as described in Materials and methods. The results are shown as a mean \pm SD ($n=3$); * $P<0.05$, significant difference between CTD-treated groups and the control as analyzed by Student's t-test.

and the results are shown in Fig. 3A-C. Fig. 3A shows that CTD increased ROS production at 2-6 h of treatment. Furthermore, CTD induced Ca^{2+} production (Fig. 3C) from 1-9 h of treatment in A375.S2 cells and these effects are time-dependent. However, Fig. 3B indicates that CTD decreased the levels of $\Delta\Psi_m$ at 24-h of treatment and shows that CTD-induced apoptosis of A375.S2 cells is associated with dysfunction of mitochondria.

CTD affects the activities of caspase-8, -9 and -3 in A375.S2 cells. To confirm whether CTD induced apoptosis through the activation of caspase-8, -9 and -3 in A375.S2 cells, cells were

pre-treated with or without the inhibitors (Z-VAD-FMK, Z-IETD-FMK, Z-LEHD-FMK and Z-DEVD-FMK: caspase-pan, -8, -9 and -3, respectively) and then were treated with $4 \mu\text{M}$ of CTD and were harvested and assessed by flow cytometric assay and the results are shown in Fig. 4A-D. Results from Fig. 4A-C indicate that CTD increased the activities of caspase-8, -9 and -3 and these effects are time-dependent. Cells were pre-treated with the inhibitors of caspase-pan, -8, -9 and -3 and then were treated with CTD and the total percentage of viable cells were measured and the results (Fig. 4D) show increased percentage of viable cells when compared to the treatment without the inhibitor.

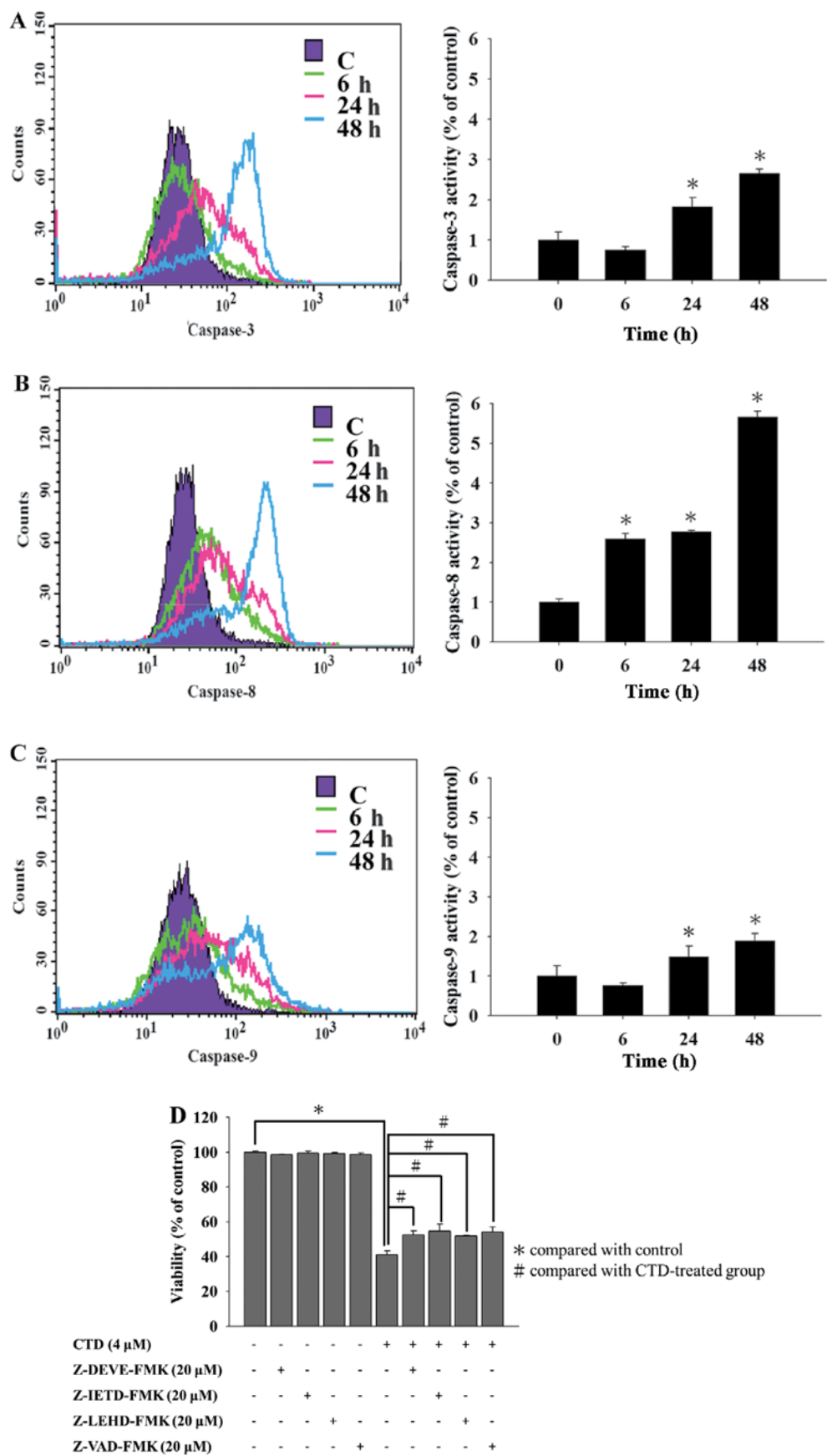


Figure 4. Cantharidin (CTD) affects caspase-3, -8 and -9 activities in A375.S2 cells. A375.S2 cells (2×10^5 cells/well) were pre-treated with or without Z-VAD-FMK, Z-IETD-FMK, Z-LEHD-FMK and Z-DEVD-FMK (inhibitors of caspase-pan, -8, -9 and -3, respectively) then were incubated 4 μ M CTD for different time periods. After harvesting and washing, the cells were re-suspended in 50 μ l of 10 μ M substrate solution of caspase-8, -9 and -3 substrates (CaspaLux8-L1D2, CaspaLux9-M1D2 and PhiPhiLux-G1D2), respectively, then the activities of (A) caspase-3, (B) caspase-8, (C) caspase-9 and (D) percentage of viable cells were measured by using flow cytometry as described in Materials and methods. The results are shown as a mean \pm SD (n=3); *P<0.05, significant difference between CTD-treated groups and the control as analyzed by Student's t-test.

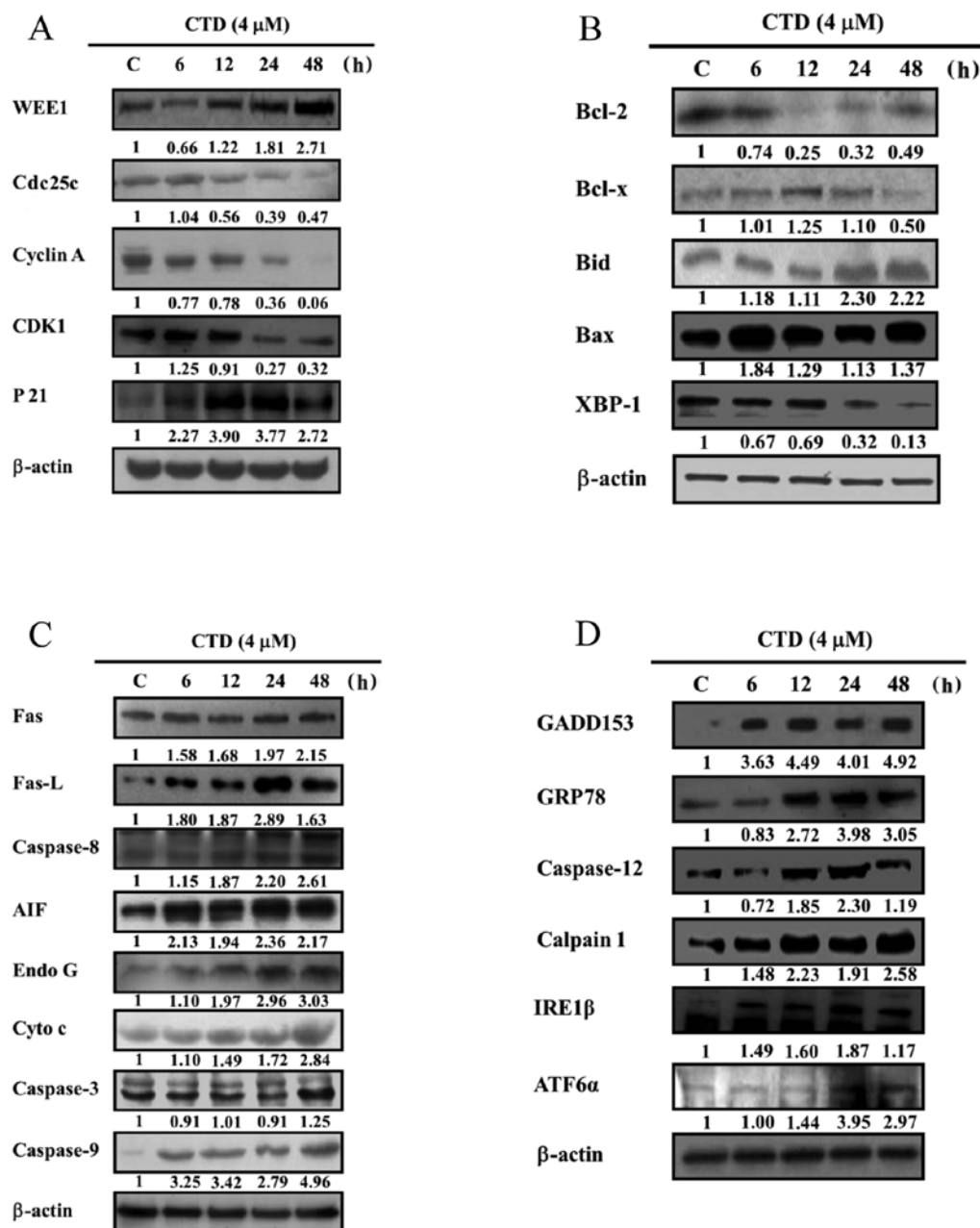


Figure 5. Cantharidin (CTD) affects G2/M phase and apoptosis-associated protein expression in A375.S2 cells. A375.S2 cells were treated with 4 μ M of CTD for 0, 6, 12, 24 and 48 h and then total proteins were quantitated and apoptosis-associated proteins were examined by western blotting as described in Materials and methods. (A) WEE1, Cdc25c, Cyclin A, CDK1 and p21; (B) Bcl-2, Bcl-x, Bid, Bax and XBP-1; (C) Fas, Fas-L, caspase-8, AIF, Endo G, cytochrome c, caspase-3 and -9; (D) GADD153, GRP78, caspase-12, calpain 1, IRE1 β and ATF6 α . β -actin, control.

These results showed that CTD induced apoptosis via the caspase-dependent pathway.

CTD affects G2/M phase arrest and apoptosis-associated protein expression in A375.S2 cells. To further investigate whether CTD induced G2/M phase arrest and apoptosis in A375.S2 cells through the presented alterations of G2/M phase and apoptosis-associated protein, cells were treated with 4 μ M of CTD for 0, 6, 12, 24 and 48 h and then the associated protein alterations were examined by western blotting and the results are shown in Fig. 5A-D. Fig. 5A indicates that CTD inhibited Cdc25c, Cyclin A and CDK1 but increased p21 and WEE1 proteins in A375.S2 cells. These cellular proteins were

known to respond to G2/M phase in cell cycle progression. We inferred that CTD could bring about the proteolytic activations of various G2/M phase proteins to obstruct the cell cycle progression of A375.S2 cells. Results in Fig. 5C show that CTD significantly increased the expression of Fas, Fas-L, and caspase-8 that is associated with the extrinsic pathway. Furthermore, results show that CTD increased cytochrome c, AIF, Endo G, caspase-9 and -3 (Fig. 5C), increased Bid, Bax, but decreased the levels of Bcl-2, Bcl-x and XBP-1 (Fig. 5B) that are all associated with the intrinsic apoptotic pathway. Additionally, Fig. 5D indicates that CTD increased ER stress-associated protein expression such as GADD153, GRP78, IRE1 β , Calpain 1, ATF6 α and

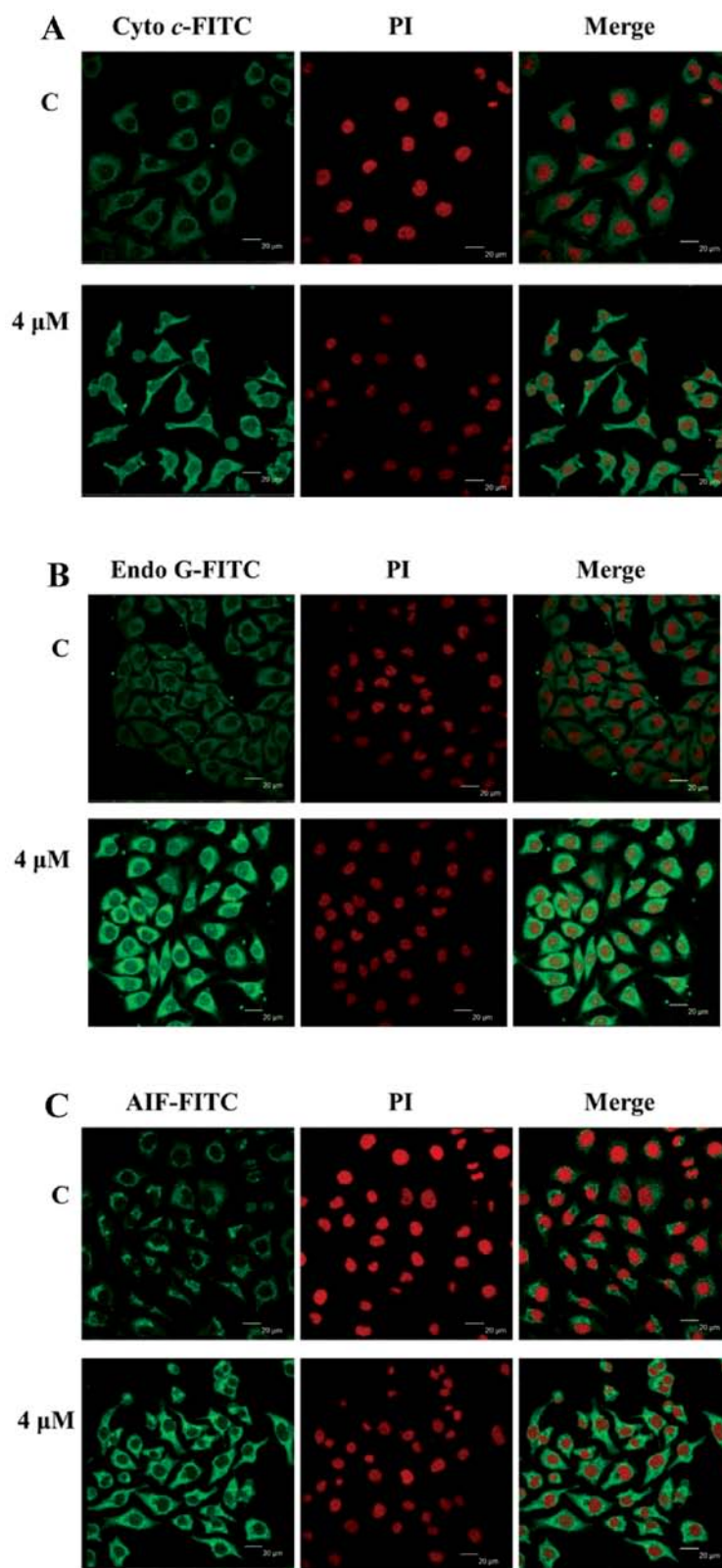


Figure 6. Cantharidin (CTD) affects the translocation of apoptosis-associated proteins in A375.S2 cells. A375.S2 cells were treated with 4 μ M of CTD for 48 h and cells were stained with (A) anti-cytochrome *c*, (B) Endo G and (C) AIF then stained with secondary antibody (FITC-conjugated goat anti-mouse IgG, green fluorescence) and were examined and photographed by a Leica TCS SP2 confocal laser microscopic systems as described in Materials and methods.

caspase-12. These results indicate that CTD induced G2/M phase arrest via inhibited cell cycle progression-associated protein and induced apoptosis through the extrinsic, intrinsic and ER stress pathways in A375.S2 cells.

CTD affected the translocation of apoptotic associated proteins in A375.S2 cells. To further confirm that CTD affects the translocation of cytochrome *c*, AIF, and Endo G involved in apoptosis in A375.S2 cells, cells were treated with 4 μ M

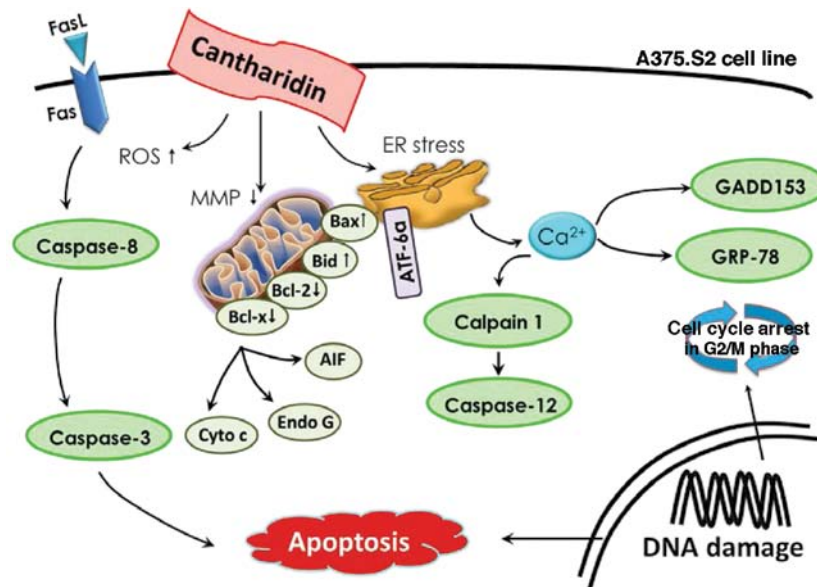


Figure 7. The proposed signaling pathways for cantharidin-induced G2/M phase arrest and apoptosis in A375.S2 human skin cancer cells.

of CTD for 48 h and then stained by anti-cytochrome *c*, AIF and Endo G to examine and photograph by confocal laser microscopic systems. Results show that CTD promoted Endo G (Fig. 6B), cytochrome *c* (Fig. 6A), AIF (Fig. 6C) releases from mitochondria in A375.S2 cells when compared to untreated (control) groups that indicated CTD induced apoptosis via the mitochondria-dependent pathway.

Discussion

It was reported that >50% of anti-cancer drugs used in patients are directly or indirectly derived from natural plants (24). CTD, a natural active compound isolated from various insects, was found to have *in vitro* antitumor activity against many human cancer cell lines (10-17). In this study, for the first time, we demonstrated that CTD induced G2/M phase arrest via the inhibition of Cdc25c and cyclin A and induced apoptosis was through death receptor (extrinsic), intrinsic (mitochondria) and ER stress pathways in A375.S2 cells. Furthermore, results indicated that CTD induced cell morphological changes (Fig. 1A) and decreased the percentage of viable cells (Fig. 1B) via the induction of G2/M phase arrest, sub-G1 phase (apoptosis) (Fig. 2).

It is well known that cells undergo cell cycle from G0/G1, S, and G2/M phase that are controlled by checkpoint-associated proteins (23,25) and agents including anticancer drugs can affect checkpoint proteins distributing the progression of cell cycle then leading the cells to undergo apoptosis (26,27). Herein, we found CTD induced G2/M phase arrest in A375.S2 cells, it also inhibited the protein expression of Cdc25c, Cyclin A and CDK1 (Fig. 6A) that are associated with G2/M arrest.

It is well documented that the induction of apoptosis triggered by anticancer drugs has been recognized as the best strategy for anticancer therapy (28,29). It was reported that intracellular ROS generation plays an important role in physiological and pathological processes. Furthermore, higher

ROS is involved in apoptotic cell death (30). Mitochondria plays a critical role in cell apoptosis (31,32) and has been suggested to act as the central executioner in apoptotic signaling pathways (33). We found that CTD increased the production of ROS (Fig. 3A) time-dependently and decreased the levels of $\Delta\Psi_m$ (Fig. 3B) in A375.S2 cells. It was reported that the mitochondria-derived ROS is caused by the dysfunction of mitochondrial electron transport chain (34). These observations indicated the mitochondrial dysfunction occurred during CTD-induced A375.S2 cell apoptosis. At 48-h of CTD treatment, it led to mitochondria dysfunction and results from western blotting also showed that CTD increased the release of cytochrome *c*, AIF and Endo G (Fig. 5C) release. Furthermore, it increased Bid and Bax but decreased Bcl-2 and Bcl-x (Fig. 5B) in A375.S2 cells. Bcl-2 gene family is divided mainly into the Bax, Bcl-2, and Bid proteins. Bax is an apoptosis-promoting protein, while Bcl-2 is an anti-apoptotic protein that plays a critical role in regulating cell apoptosis (35,36). By western blotting we found that the expression of Bax was increased and that of Bcl-2 was reduced in A375.S2 cells when treated with CTD, therefore increasing the Bax/Bcl-2 ratio significantly.

Other studies have shown that oxidative stress stimulates translocation of Bax from cytosol to mitochondria causing cytochrome *c* release inside the cytoplasm during liver apoptosis (37). CTD-induced ROS generation, and we suggest that CTD-induced apoptosis might be modulated by the ROS-mediated pathways in A375.S2 cells.

It was well known that cysteine-containing aspartate-specific proteases (caspases) are involved in cell apoptosis (38,39). Caspase-8 is related to extrinsic pathway and caspase-9 is involved in the intrinsic pathway, however, caspase-3 is related to the common pathway of cell apoptosis and it is a key executor of cell apoptosis. In our study, the results in Fig. 5 show that increased activation of caspase-8, -9 and -3 (Fig. 4), and expression of protein (Fig. 6B) are associated with cell apoptosis. Furthermore, cells were pre-treated with

the inhibitors of caspase-8, -9 and -3 and then treated with CTD leading to increase in the percentage of viable cells when compared to CTD only treated cells.

In conclusion, caspase-pathway activation, mitochondria dysfunction and oxidative stress (ROS generation) induced by CTD contribute to the activation of the apoptotic pathway in CTD-treated A375.S2 cells. Furthermore, the modulating expression and translocation of apoptotic proteins induced the mitochondrial pathways in A375.S2 cells as shown in Fig. 7. Based on these observations, CTD inhibits human skin cancer A375.S2 cellular growth and our studies provide a better understanding of the molecular mechanism of CTD function.

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