Cantharidin induces apoptosis in human bladder cancer TSGH 8301 cells through mitochondria-dependent signal pathways

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Abstract. Cantharidin has shown potent anticancer activities on many types of human cancer cells. This study was performed to elucidate whether mitochondria and caspases are involved in the modulation of apoptosis and cell cycle arrest by cantharidin in human bladder cancer cells. The effect of cantharidin on cell cycle arrest, apoptosis, caspases, reactive oxygen species (ROS) and mitochondrial membrane potential ($\Delta\Psi_m$) were measured by flow cytometry, and the levels of apoptosis-associated proteins and its regulatory molecules were studied by Western blotting. Cantharidin-induced apoptosis and DNA damage was determined by flow cytometric analysis, DAPI staining and Comet assay. After cantharidin treatment, the active forms of caspase-3, -8 and -9 were promoted. Cantharidin-induced apoptosis was associated with enhanced ROS and Ca^{2+} generations, caused DNA damage, decreased the levels of $\Delta\Psi_m$ and promoted Endo G and AIF released from mitochondria. Cantharidin-induced G0/G1 arrest was associated with a marked decrease in the protein expressions of cyclin E and Cdc25c but promoted the levels of p21 and p-p53. Cantharidin-induced apoptosis was accompanied with up-regulation of the protein expression of Bax and PARP, but down-regulation of the protein levels of Bcl-2, resulting in dysfunction of mitochondria then led to Endo G and AIF release for causing induction of apoptosis.

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Introduction

Bladder cancer is one of the leading causes of death in the world. Based on the 2008 report from the Department of Health, R.O.C. (Taiwan) indicated that 3.0 individuals per 100,000 die annually from bladder cancer in Taiwan. The current treatment modalities are inadequate. Therefore, novel therapies are needed to reduce the effects of the increasing incidence of bladder cancer. Numerous studies have been shown that cytotoxic agents and DNA damaging agents can induce cell cycle arrest and induce apoptotic cell death (1-3).

Apoptosis is a highly regulated mechanism leading cells to undergo programmed cell death through the extrinsic and the intrinsic pathways (4,5). The extrinsic pathway is triggered by the interaction between specific ligands and surface receptors (6) of cells such as CD95/Fas, tumor necrosis factor (TNF) and death receptors (5). The intrinsic pathway is triggered by various stimuli such as DNA damage, cellular distress, hypoxia and cytotoxic agents, which act inside the cell (5). The third apoptotic pathway is called endoplasmic reticulum (ER) stress pathway (7) which is triggered by the interaction between the mitochondria and ER which plays an essential role in ER stress-mediated cell death. Recently, it was reported that apoptosis can be divided into caspase-dependent and -independent pathways. The caspase activations are often regulated by various cellular proteins, in particular, the members of the inhibitor of apoptosis such as IAP (8) or Bcl-2 families (9,10). Currently, the cure for bladder cancer including surgery, radiotherapy and chemotherapeutic options, however, they are still inadequate, many studies are focused on identifying new agents and novel targets for treating bladder cancer. To induce apoptosis of cancer cells is one of the best strategies for treatment of cancer by chemotherapeutic agents.

Cantharidin, a type of terpenoid, is a derivative of *Blister Beetles* belonging to the order of *Coleoptera* and the family of *Meloidae* (11), and it is used in Chinese medicine (12). Cantharidin was reported to induce cell cycle arrest (13) and apoptosis in many human cancer cells such as hepatoma

(14), colon cancer (15), bladder carcinoma (16), breast cancer (17) and oral buccal carcinoma and leukemia cells (18). Also it was reported that cantharidin induced cell cycle arrest in the G2/M phase and induced apoptosis in human bladder cancer T24 cells. In addition, cantharidin also stimulated COX2 over-expression and PGE2 production in T24 cells, in a dose-dependent manner (16). However, there is no available information to address the effects of the cantharidin on human bladder carcinoma TSGH 8301 cells. Therefore, in this study, we selected human bladder carcinoma TSGH 8301 cells for examining the effects of cantharidin on cell cycle arrest and apoptosis and results showed that cantharidin potently induced cell cycle arrest and apoptosis in TSGH 8301 cells. The results showed that cantharidin induced G2/M arrest and apoptosis which is mitochondrial-dependent and via JNK signal pathways offering new information to show cantharidin-induced apoptosis in bladder carcinoma TSGH 8301 cells.

Materials and methods

Chemicals and reagents. Cantharidin, dimethyl sulfoxide (DMSO), potassium phosphates, propidium iodide (PI), ribonuclease-A, Tris-HCl, Triton X-100 and trypan blue were purchased from Sigma Chemical Co. (St. Louis, MO, USA). RPMI-1640 medium with 2 mM L-glutamine + 10% fetal bovine serum, penicillin-streptomycin and trypsin-EDTA were obtained from Gibco BRL (Grand Island, NY, USA).

Cell culture. The human bladder carcinoma TSGH 8301 cell line were obtained from the Food Industry Research and Development Institute (Hsinchu, Taiwan). TSGH 8301 cells were cultured on 75 cm^2 tissue culture flasks with RPMI-1640 medium (Gibco BRL). The cell medium with 2 mM L-glutamine was adjusted to contain 10% fetal bovine serum (Gibco BRL), and 1% penicillin-streptomycin (100 U/ml penicillin and 100 μg/ml streptomycin) (19) and grown at 37˚C under a humidified 5% CO_2 atmosphere.

Determination of cell morphology, viability, cell cycle and apoptosis. TSGH 8301 cells were seeded at a concentration of $5x10^5$ cells/well in a 12-well plate for 24 h, then were incubated with 0, 5, 10, 15, 20 and 25 μM of cantharidin at 37˚C, 5% CO_2 and 95% air for 24 h. Cells were examined and photographed by phase-contrast microscope for the examination of morphological changes. Then cells were harvested by centrifugation and were stained with PI (5 μg/ml) then analyzed by flow cytometry (Becton-Dickinson, San Jose, CA, USA) for viability measurements as previously described (19-23). For cell cycle distribution and sub-G1 determination, isolated cells were fixed gently by 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 dark room for 30 min at 37˚C, then were analyzed with a flow cytometer equipped with an argon ion laser at 488 nm wavelength (19-23).

DAPI staining. For DAPI staining, approximately $5x10^4$ cells/ml of TSGH 8301 cells were treated with 0, 2.5, 5, 10 and 15 μM of cantharidin for 24 h. Cells were stained with DAPI (4,6-diamidino-2-phenylindole dihydrochloride), then examined and photographed using a fluorescence microscope as previously described (19-23).

Detections of ROS (reactive oxygen species), Ca^{2+} and mitochondrial membrane potential ($\Delta\Psi_m$). Approximately $2x10^5$ cells/ml of TSGH 8301 cells were treated with 10 μM of cantharidin for 0.5, 1, 3, 6 and 12 h then cells from each treatment were harvested and re-suspended in 500 μl of 2,7-dichlorodihydrofluorescein diacetate (DCFH-DA) (10 μM) for ROS, in 500 μl of 1-[2-amino-5-(6-carboxyindol-2-yl) phenoxy]-2-(2'-amino-5' methylphenoxy)ethane-*N*,*N*,*N'*,*N'*-tetra acetic acid pentaacetoxymethyl ester (Indo 1/AM) (3 μg/ml) for Ca^{2+} and in 500 μl of $DiOC_6$ (4 μmol/l) for $\Delta\Psi_m$. Cells was incubated at 37˚C for 30 min before being analyzed by flow cytometry (19-23).

Western blotting of cell cycle and apoptosis-associated proteins. Approximately $1x10^7$ cells of TSGH 8301 cells were treated with 10 μM cantharidin for 0, 6, 12, 24 and 48 h. Cells were harvested and lysed with lysis buffer containing 40 mM Tris-HCl (pH 7.4), 10 mM EDTA, 120 mM NaCl, 1 mM dithiothreitol, 0.1% nonide P-40 and the total proteins were quantitated as described previously (19-23) and the total proteins (30 μg) were used for Western blot analysis and all samples were performed using 10% Tris-glycine-SDS-polyacrylamide gels for 30 min then the proteins were transferred to a nitrocellulose membrane by electroblotting. Then they were incubated with primary antibodies such as (A) cyclin E, Cdc25c, p21 and p-p53 (Santa Cruz Biotechnology, CA); (B) Bal-2, Bax, caspase-9, AIF, Endo G, caspase-3, PARP and caspase-8 (R&D Systems, Minneapolis, USA), washed and incubated with secondary antibody for enhanced chemiluminescence (NEN Life Science Products, Inc., Boston, MA, USA) as described previously (19-23). As a loading control, we used anti-ß-tubulin (a mouse monoclonal antibody).

Confocal laser scanning microscopy for protein translocation. TSGH 8301 cells at density of $5x10^4$ cells/well were cultured on 4-well chamber slides then were treated without or with 5 μM catharidin for 24 h. Then cells on the slides were fixed in 4% formaldehyde in PBS for 15 min, permeabilized with 0.3% Triton-X 100 in PBS for 1 h with blocking of non-specific binding sites using 2% BSA as described previously (24). Primary antibodies to AIF and Endo G (1:100 dilution) (green fluorescence) were used to stain the fixed cells for overnight then were washed twice with PBS and were stained with secondary antibody (FITC-conjugated goat anti-mouse IgG at 1:100 dilution), and followed by DNA staining with mitotracker (red fluorescence) as described previously (24). All samples were photomicrographed and were obtained using a Leica TCS SP2 confocal spectral microscope (24).

Statistical analysis. The results are rported as mean ± SD and the difference between the cantharidin-treated and control groups were analyzed by Student's t-test, a probability of $p<0.05$ being considered significant.

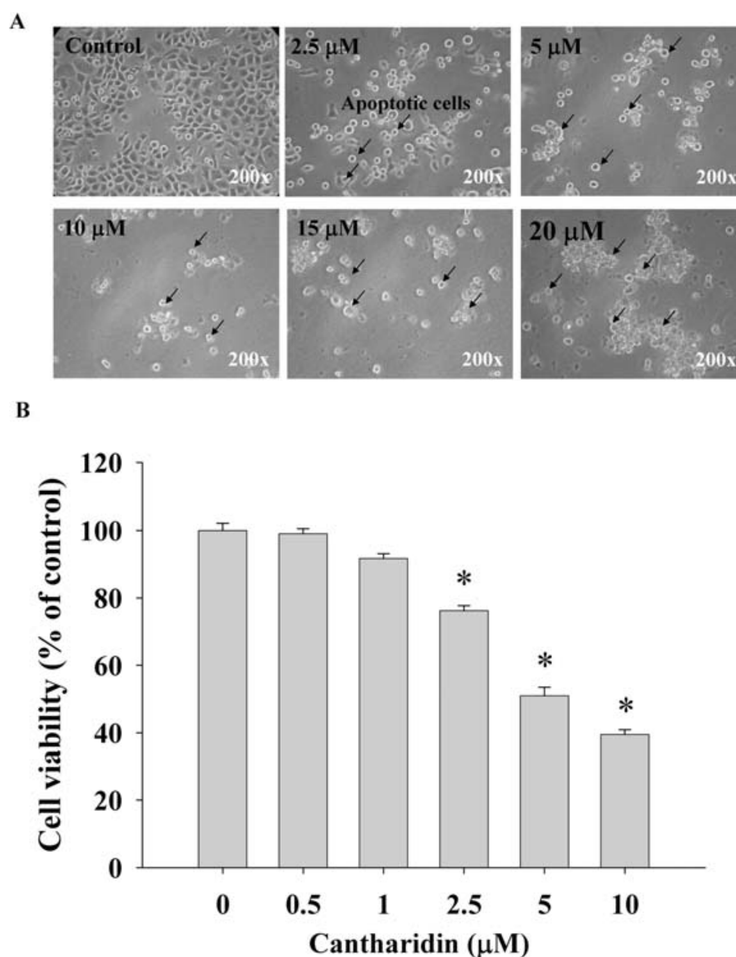

Figure 1. Cantharidin affects the morphology and viability of human bladder cancer TSGH 8301 cells. Cells were treated with different doses of cantharidin for 24 h, then cells were examined and photographed for morphological changes (A) by phase-contrast microscope. Cells were harvested for determination of percentage of viable cells (B) by using flow cytometeric assay as described in Materials and methods. Significantly different from the 0 h treatment (control) at $^{*}p<0.05$.

Results

Effects of cantharidin on TSGH 8301 cell morphology, viability and cell cycle detected by flow cytometry. We determined growth inhibition effects of cantharidin on the morphological changes under phase-contrast microscope, viability and cell cycle distribution by using flow cytometric assay, and the results are shown in Figs. 1A and B, and 2A and B. Increasing the dose of cantharidin and/or time of incubation led to the increase of morphological changes (Fig. 1A) and the decrease of the percentage of viability (Fig. 1B). Cantharidin at 5 μM decreased by almost 45% the viable cells at 48 h treatment. Fig. 2A and B show that TSGH 8301 cells were exposed to various doses of cantharidin for 24 h incubation increasing the percentage of cells in G0/G1 dose-dependently. The sub-G1 groups (apoptosis) also appeared in the cell cycle distribution.

Effects of cantharidin on DNA damage and apoptosis in TSGH 8301 cells. To investigate cantharidin-induced apoptosis via DNA damage, the TSGH 8301 cells were treated with cantharidin, cells were isolated for DAPI staining and/or cells were stained with Annexin V for apoptosis determination. The results are presented in Fig. 3A and B, which indicate that cantharidin induced DNA condensation and decreased the cell number (Fig. 3A) and induced apoptosis (Fig. 3B), and those effects are dose-dependent.

Effects of cantharidin on the levels of reactive oxygen species (ROS), Ca^{2+} and mitochondrial membrane potential ($\Delta\Psi_m$) in TSGH 8301 cells. For investigating whether cantharidin-induced apoptosis is via the mitochondrial pathway, TSGH 8301 cells were treated with 5 μM cantharidin for various time periods, the levels in ROS and Ca^{2+} productions and $\Delta\Psi_m$ were measured and determined by flow cytometric assay and the results are shown in Fig. 4A-C. These results indicate that cantharidin treatment in TSGH 8301 cells led to an increase in the productions of ROS (Fig. 4A) and Ca^{2+} (Fig. 4B) and it also induced a decrease of $\Delta\Psi_m$ (Fig. 4C). These effects are time-dependent.

Effects of cantharidin on the apoptotic-associated protein translocation in TSGH 8301 cells. For investigating whether cantharidin affected the apoptotic-associated protein translocation in TSGH 8301 cells, the cells (5×10^4 cells/well) were plated on 4-well chamber slides, treated with 5 μM

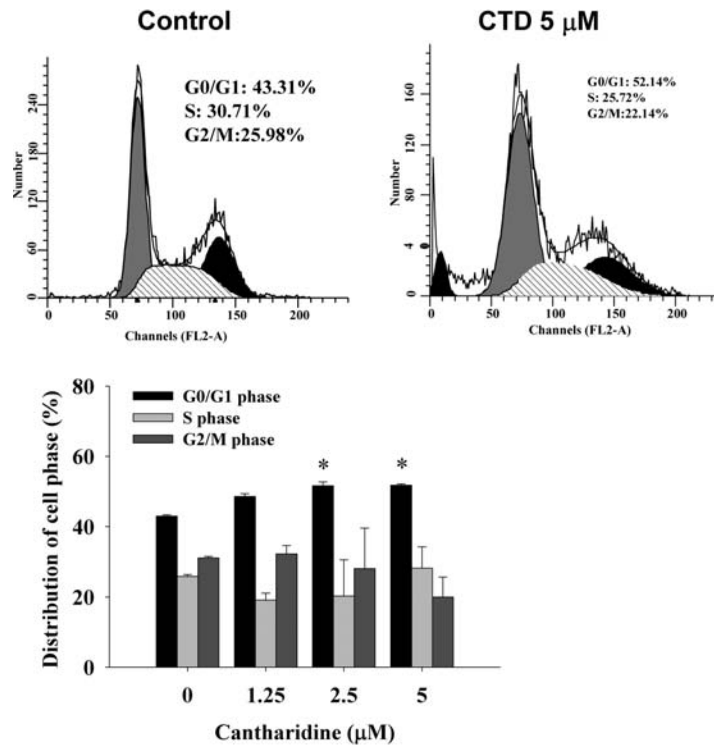

Figure 2. Cantharidin induces cell cycle arrest in TSGH 8301 cells. Cells were treated with 0, 2.5, 5 and 10 μM of cantharidin for 24 h then cells were harvested for examinations of cell cycle distribution by flow cytometry as described in Materials and methods. (A) Representative profiles; (B) percentage of cell distribution. Significantly different from the 0 h treatment (control group) at $^{*}p<0.05$.

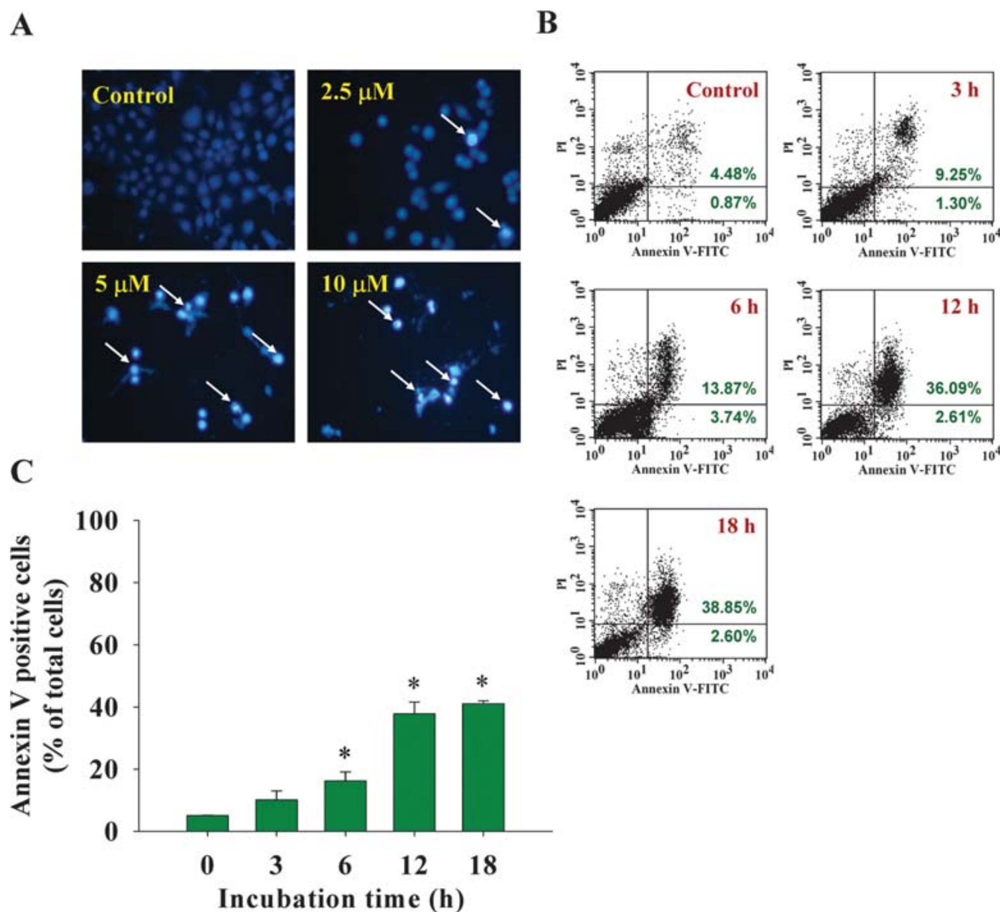

Figure 3. Cantharidin induces DNA damage and apoptosis in TSGH 8301 cells. Cells were treated with 0, 1.25, 2.5 and 5 μM of cantharidin for 24 h or with 5 μM of cantharidin for 0, 3, 6, 12 and 18 h, then cells were harvested for examinations of DNA damage by DAPI staining (A) or apoptosis by Annexin V staining and were analyzed by flow cytometry (B) as described in Materials and methods. The percentage of apoptosis was calculated (C). Significantly different from the 0 h treatment (control group) at $^{*}p<0.05$.

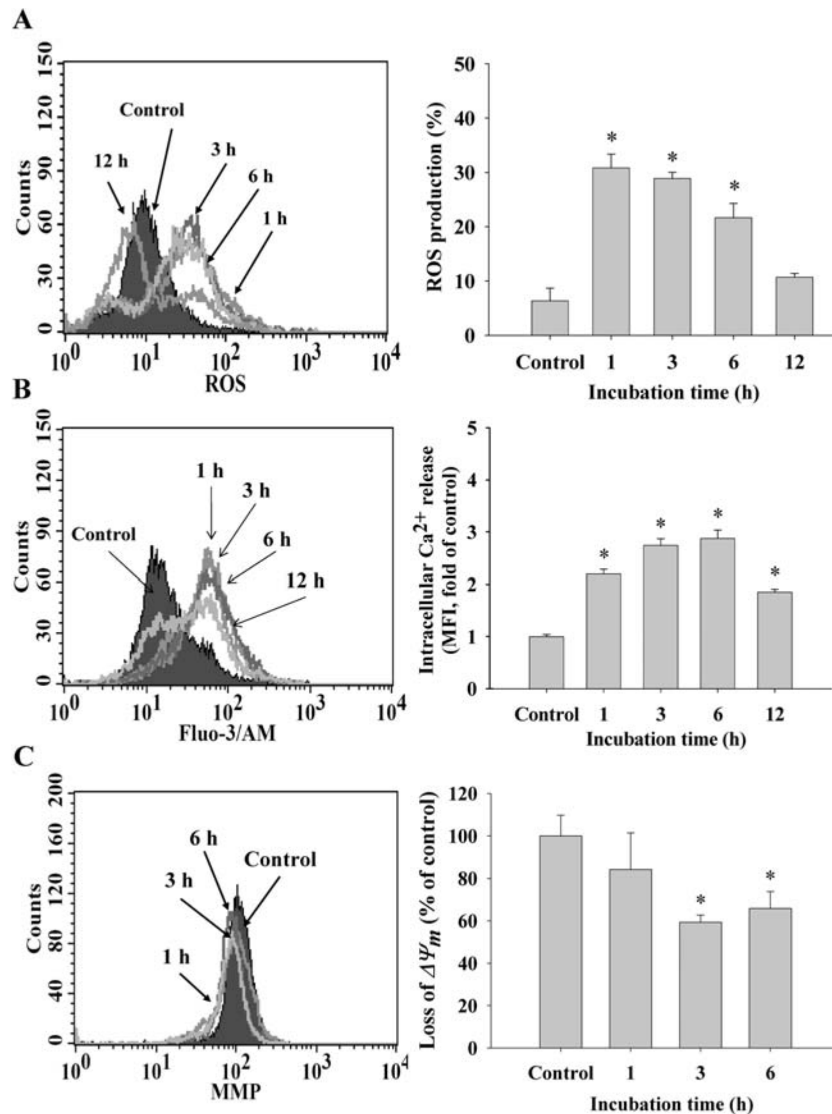

Figure 4. Cantharidin induced reactive oxygen species (ROS) and Ca^{2+} productions, and affected the levels of mitochondrial membrane potential ($\Delta\Psi_m$) in TSGH 8301 cells. TSGH 8301 cells ($5x10^5$ cells/ml) were treated with 5 μM of cantharidin for different time periods. Cells were harvested for the percentage of ROS (A), Ca^{2+} (C) and $\Delta\Psi_m$ (B) that were stained by DCFH-DA, $DiOC_6$ and Indo-1/AM, respectively. The stained cells were determined by flow cytometry as described in the Materials and methods. Values are means ± SD (n=3). Significantly different from the 0 h treatment (control group) at $^*p<0.05$.

cantharidin for 24 h, stained by antibody, then examined and photographed by confocal laser microscopic systems. The results are shown in Fig. 5A and B, which indicate that Endo G (Fig. 5A) and AIF (Fig. 5B) are released from mitochondria then translocated to the nuclei. From the double- immunofluorescence labeling it was clearly confirmed by means of confocal scanning microscopy that Endo G and AIF were increased after exposed to cantharidin in TSGH 8301 cells.

The effects of cantharidin on the associated protein levels of apoptosis in TSGH 8301 cells. For investigating cantharidin-induced apoptosis through the possible signaling pathways in TSGH 8301 cells, cells were treated with 10 μM of cantharidin for various time periods then protein levels were analyzed by Western blotting. The results are shown in Fig. 6, where cantharidin increased p21 and p-p53 but decreased the levels of cyclin E and Cdc25c that led to G0/G1 phase arrest (Fig. 6A), however, cantharidin increased the levels of Bax, cleavage-caspase-8, -9, -3, AIF Endo G and PARP (Fig. 6B) protein levels but decreased cytosolic Bcl-2 (Fig. 6B) protein levels in TSGH 8301 cells, leading to apoptosis. We suggest that cantharidin-induced apoptosis is mediated by the mitochondrial and ER stress pathways.

Discussion

Natural products and their derivatives such as instance, vinca alkaloids, taxanes and camptothecins, have been used commonly in cancer chemotherapy (25), however, the majority of natural products come from plants, but animal studies are few. Substantial evidence has shown that cantharidin induced cell cycle arrest and apoptosis in many human cancer cell lines including human bladder T24 cancer cells. In particular, cantharidin induced apoptosis via active caspase-3 in T24 cells (16). In the present study, we used the human bladder

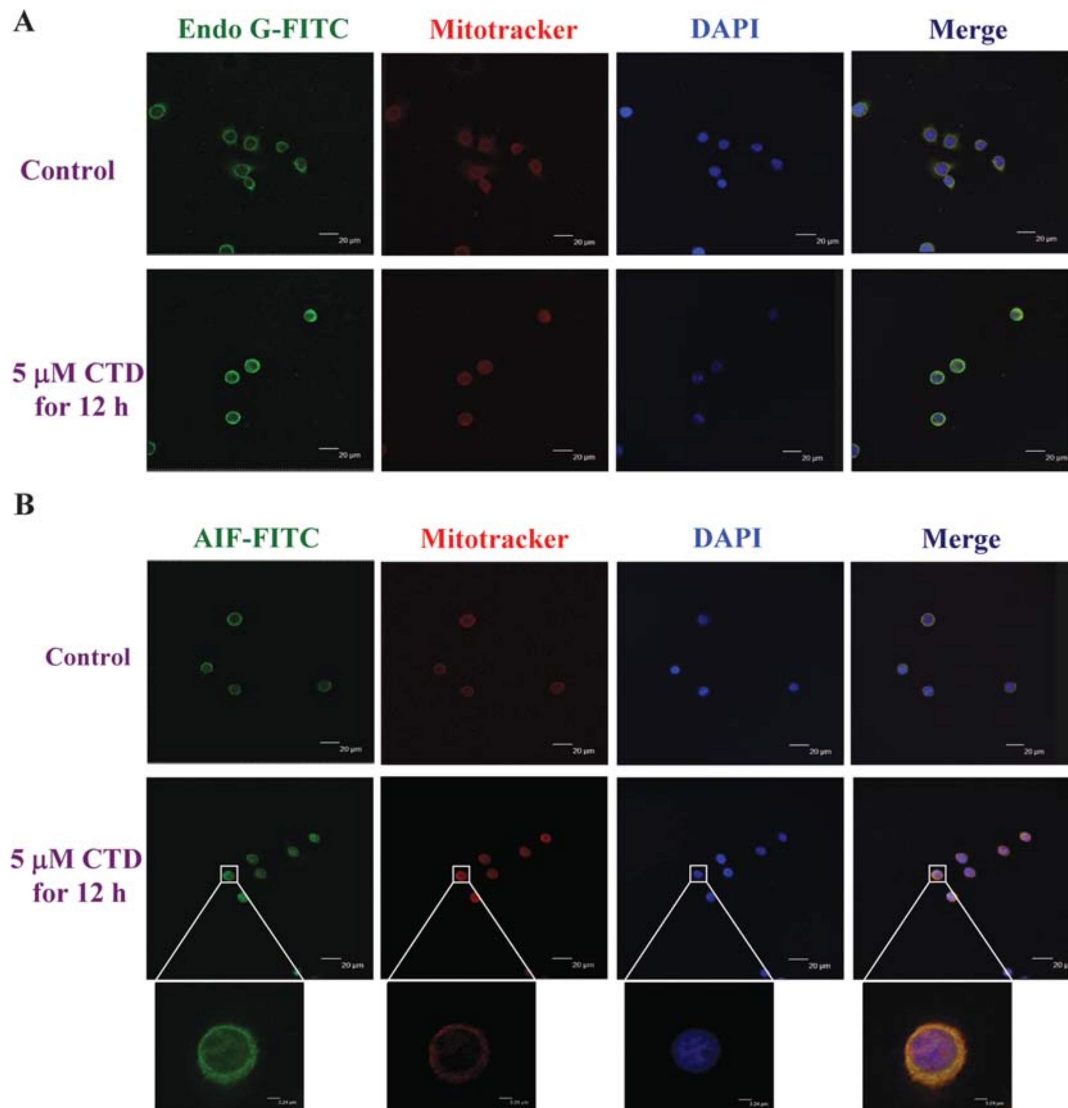

Figure 5. Cantharidin affected Endo G and AIF distribution in TSGH 8301 cells. Cells were incubated with cantharidin at 5 μM for 12 h and then fixed and stained with primary antibodies to Endo G (A) and AIF (B) before FITC-labeled secondary antibodies were used (green fluorescence) and the proteins were detected by a confocal laser microscopic system. The nuclei were stained by mitotracker (red fluorescence). Areas of colocalization between AIF and Endo G expressions and cytoplasm and nuclei in the merged panels are yellow. Scale bar, 40 μm.

cancer TSGH 8301 cells after exposure to different concentrations of cantharidin and results showed that cantharidin induced apoptosis through mitochondria-dependent and JNK signal pathways. Therefore, these novel findings offer more information regarding cantharidin-induced apoptosis in human bladder cancer cells. Furthermore, it was reported that cantharidin induces selective cytotoxicity in pancreatic cancer cells with less toxicity in normal pancreatic duct cells (26).

In this study, we investigated the antitumor activity of cantharidin on bladder cancer TSGH 8301 cells. Treatment with cantharidin induced substantial growth inhibition in TSGH 8301 cells (Fig. 1). Further investigation was done for the molecular mechanisms associated with the inhibitory role of cantharidin including cell cycle arrest, apoptosis and the role of mitochondria and results showed that: i) Cantharidin treatment resulted in a marked G0/G1 phase cell cycle arrest (Fig. 2). Western blotting showed that cantharidin increased the levels of p53 and p21 but decreased the levels of Cdc25c and cyclin E that may lead to G0/G1 phase arrest (Fig. 6). Cell cycle is controlled by distinct protein kinase complexes such as cyclins that are necessary for cdc/cdk kinase activity (27). Progression through G1 involves the activation of cyclin D/cdk2, 4, 5 and 6; G1/S involves the activation of cyclin E/cdk2; S involves the activation of cyclin A/cdk2, G2/M is regulated by cyclins A and cyclin B/cdk1 (28,29). ii) Similar to previous reports on other cancer cells, we found that cantharidin induced apoptosis and promoted the active form of caspase-8, -3 and caspase-9 (Fig. 6B), however, we showed that cantharidin decreased the levels of $\Delta\Psi_m$ which are involved in AIF, Endo G release from mitochondria, that was the novel finding and may suggest that cantharidin induced apoptosis also through caspase-dependent, -independent and mitochondrial-dependent pathways. Western blotting also showed that cantharidin promoted the expression of Bax (pro-apoptotic protein) and decreased the levels of Bcl-2 (anti-apoptotic protein) (Fig. 6B). This is in agreement with other reports indicated that cantharidin

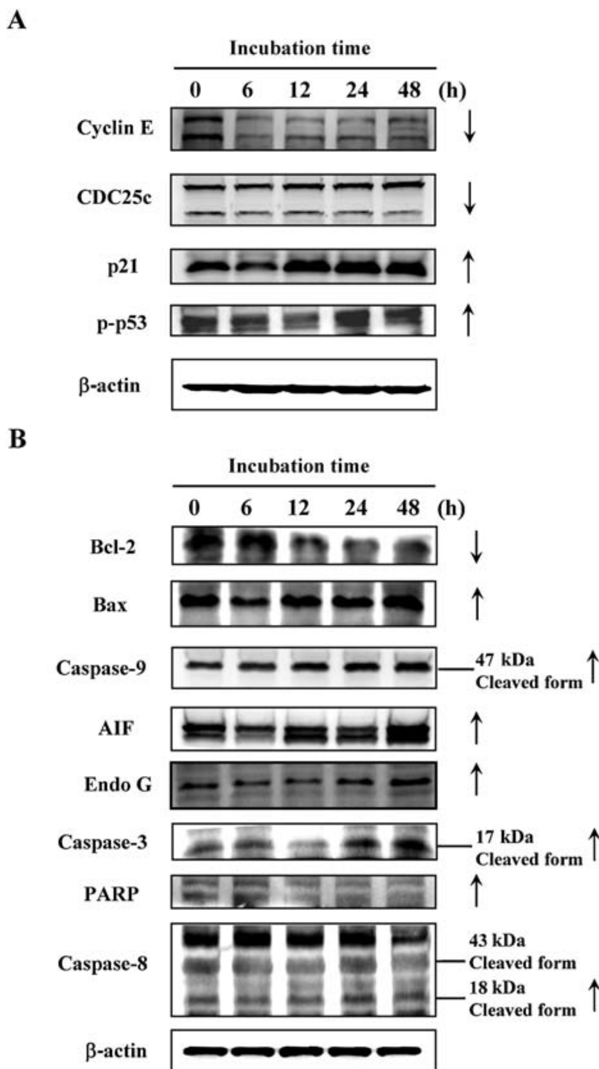

Figure 6. Cantharidin affects the proteins levels of G0/G1 arrest and apoptosis in TSGH 8301 cells. Cells were treated with 5 μM of cantharidin for 0, 6, 12, 24 and 48 h then the total proteins were prepared and detected by Western blotting as described in Materials and methods. Respectively, primary antibodies for (A) cyclin E, Cdc25c, p21, p-p53, (B) Bcl-2, Bax, caspase-9, AIF, Endo G, caspase-3, PARP and caspase-8 were examined by Western blotting.

treatment led to dramatically decreased expression of Bcl-2 (16,30). Other reports have shown that some Bcl-2 family members (such as Bax, Bcl-XL, Mcl-1, Bcl-2 and Bid) that are located on the mitochondrial membrane can alter the permeability of the mitochondrial membrane and then led to the activations of caspases followed by apoptotic cell death (9-10,31). iii) Our results also showed that cantharidin promoted the productions of ROS and Ca^{2+} in TSGH 8301 cells (Fig. 4). This is in agreement with other reports indicating that the cytotoxicity of cantharidin was dependent on the induction of oxidative stress (30) which could induce subsequent apoptosis and cell cycle arrest (32). However, it was reported that cantharidin induced ROS production in human pancreatic cancer PANC-1 and CFPAC-1 cells in an oxidative stress-independent pathway (26). Furthermore, cantharidin have been showed to induce G2/M phase arrest in human bladder cancer T24 cell line (16), but we found that cantharidin induced G0/G1 phase arrest in human bladder cancer TSGH 8301 cells (Fig. 2A and B). Apparently, cantharidin-induced cytotoxicity may have cell type specificity. Further investigations are needed.

Furthermore, we tried to investigate whether cantharidin-induced cytotoxicity was related to MAPK pathways in bladder cancer TSGG 8301 cells. In the present study, ERK, JNK and p38 were all found to be activated in TSGH 8301 cells when treated with cantharidin. We suggest that the cytotoxicity effect of cantharidin is dependent on the JNK pathway. It has been reported that activation of JNK can increase the expressions of p21 (33), Bad (34), Bak (35) and TNF-α (36) which were found to be escalated when treated with cantharidin, therefore, cantharidin-induced cytotoxicity could be due to the induced JNK activation and the subsequent affect associated genes for further trigger G2/M phase arrest and apoptosis in TSGH 8301 cells.

In summary, based on these observations, we have obtained convincing evidence that cantharidin efficiently inhibits the growth of human bladder cancer cells through mitochondria-dependent signal pathways as shown in Fig. 7. Further investigation on both *in vivo* and *in vitro* bladder cancer models is needed.

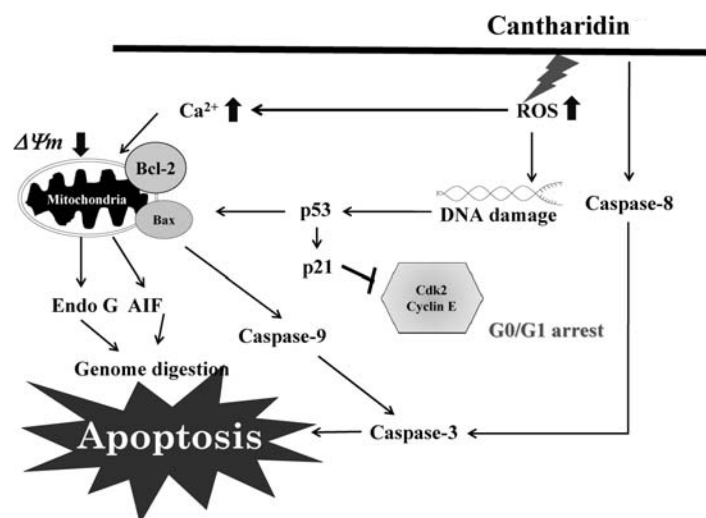

Figure 7. The proposed model of molecular signal pathways from TSGH 9301 cells after exposure to cantharidin.

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