Cantharidin induced G_2/M arrest and apoptosis in colo 205 cancer cells through CDK1 inhibition and caspase-dependent pathway

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Abstract. Cantharidin (CTD) is a Traditional Chinese Medicine and an effective component which isolated from *blister beetle*, and it has been demonstrated to have anti-cancer activity, antibiotic activity, antivirus activity and immune regulation function. It was reported that CTD induced cell cycle arrest and apoptosis in many cancer cells. However, there is no report to show CTD induced cell cycle arrest and apoptosis in human colorectal colo 205 cells. In this study, we treated the colo 205 cells with CTD and investigated its molecular mechanism. CTD induced growth inhibition, G2/M arrest and apoptosis in colo 205 cells. The IC_{50} is 20.53 μM in CTD treated colo 205 cells. DAPI/TUNNEL double staining and Annexin V confirm the apoptosis in CTD treated-colo 205 cells. CTD caused G2/M arrest, down-regulation of CDK1 activity, decreased Cyclin A, Cyclin B, CDK1 and increased CHK1 and p21 protein levels. Colorimetric assays indicated that induced activities of casapse-8, casapse-9 and casapse-3 in CTD treated colo 205 cells. We also showed that CTD promoted the production of ROS and decreased the levels of Mitochondrial membrane potential (MMP) in CTD treated colo 205 cells. Consequently, CTD-induced growth inhibition was significantly attenuated by NAC inhibitor. CTD stimulated Fas/CD95, caspase-3 active form, cytochrome *c* and Bax protein levels, but reduced the protein levels of Pro-caspase-8, Pro-caspase-9 and Bcl-2 by Western blotting analysis. We suggested that CTD is able to induce G2/M arrest and apoptosis on colo 205 cells through the CDK1 activity inhibition and caspase-dependent signal pathway.

Introduction

Colorectal cancer is the frequent reason of death for cancer in the world and in Taiwan (1). Colorectal cancer is a multistep process including progressive disruption of intestinal epithelium growth (2). The treatment of colorectal cancer in clinical consists of surgery, radiation and chemotherapy, but these treatments of human colon cancer are limited and the result is not acceptable. Induction of cell cycle arrest and apoptosis in cells may be a considered strategy for colon cancer. The process of cell cycle has been investigated frequently, particularly CDK1/Cyclin B complex play a very important role for regulation of G2/M phase (3-4). Many evidences show that through the cell arrest and apoptosis can induced the tumor cell death (5).

Apoptosis, programmed cell death, is an important in shaping an organism during embryonic development. Apoptosis causes specific morphological modification such as cell membrane blebbing, induced translocation of phosphatidylserine (PS) from inner membrane to the outer membrane, chromatin condensation, caspases activation and DNA fragmentation (Reference: Houttuynia cordata Thunb extract induces apoptosis through mitochondrial-dependent pathway in HT-29 human colon adenocarcinoma cells. Tang YJ, Yang JS, Lin CF, Shyu WC, Tsuzuki M, Lu CC, Chen YF, Lai KC. Oncol Rep. 2009 Nov;22(5):1051-6. Visconti R and Grieco D: New insights on oxidative stress in cancer. Curr Opin Drug Discov Devel 12: 240-245, 2009). Many studies have demonstrated that death-receptor, mitochondria and endoplasmic reticulum played as an important regulator in apoptosis (Reference: Murgia M, Giorgi C, Pinton P and Rizzuto R: Controlling metabolism and cell death: At the heart of mitochondrial calcium signalling. J Mol Cell Cardiol 2009. Diallyl disulfide induces apoptosis in human colon cancer cell line (COLO 205) through the induction of reactive oxygen species, endoplasmic reticulum

stress, caspases casade and mitochondrial-dependent pathways. Yang JS, Chen GW, Hsia TC, Ho HC, Ho CC, Lin MW, Lin SS, Yeh RD, Ip SW, Lu HF, Chung JG. Food Chem Toxicol. 2009 Jan;47(1):171-9. Epub 2008 Nov 12.) The activation of effector caspases such as caspase-3 and -7 by initiator caspases (caspase-8 and caspase-9) are responsible for the cleavage of cellular substrates then degrades the chromosomes into nucleosomal fragments during apoptosis (Reference: Schauer T, Tombacz I, Ciurciu A, Komonyi O and Boros IM: Misregulated RNA Pol II C-terminal domain phosphorylation results in apoptosis. Cell Mol Life Sci 66: 909-918, 200). Two major pathways are involving cell apoptosis, one is the death receptor pathway and the other is mitochondrial pathway. The death receptor pathway is involves Fas/CD95 and caspase-8 activation and then activates caspase-3. The mitochondrial pathway involves the mitochondrial changes, decrease of mitochondrial membrane potential (MMP), increase of reactive oxygen species (ROS) production,, which lead to the release of cytochrome *c*, Apaf-1, AIF and procaspase-9, and then combine with Apaf-1 and procaspase-9 to form the apoptosome (Reference: Investigation of anti-leukemia molecular mechanism of ITR-284, a carboxamide analog, in leukemia cells and its effects in WEHI-3 leukemia mice. Wen YF, Yang JS, Kuo SC, Hwang CS, Chung JG, Wu HC, Huang WW, Jhan JH, Lin CM, Chen HJ. Biochem Pharmacol. 2010 Feb 1;79(3):389-98.). Many researches have focused on selectively killing cancer cells through the induction of apoptosis.

Cantharidin (CTD) is extracted from *mylabris* (*blister beetle*, a Traditional Chinese Medicine), is a type of terpenoid, has been demonstrated to possess of antitumor, antibiotic, antivirus and regulation of immunity (6-7). CTD has been used for a medical agent over 2,000 years; there are many usage including abortifacient, dropsy, aphrodisiac, wart (6). In recently studies, CTD has inhibited many cancer cells proliferation, such as pancreatic cancer, hepatoma, bladder carcinoma, and breast

cancer (8-10). The activity of PP2A could be inhibited by CTD (8). PP2A can suppress tumor proliferation and also used a target molecules to detect the suppression of tumor (10). The purpose of this study examined the CTD inhibits proliferation and anti-cancer mechanism on human colorectal colo 205 cells.

Materials and Methods

Chemicals and reagents. Cantharidin (CTD), MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide), propidium iodide (PI), Tris-HC1, Triton X-100, N-acetylcysteine (NAC) and RNase 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-8, caspase-9 and caspase-3 were purchased from Cell Signaling Technology (Beverly, MA); antibodies for Cyclin A, Cyclin B, CDK1, CHK1, p21, cytochrome c, Bax, Bcl-2, β-actin, and horseradish peroxidase (HRP)-linked goat anti-mouse IgG, goat anti-rabbit IgG, were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Caspase-9, caspase-8 and caspase-3 activities assay kits were obtained from R&D system Inc (Minneapolis, MN, USA). Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay kit, Annexin V assays kit and 4'-6-diamidino-2-phenylindole (DAPI) were obtained from Invitrogen Corp (Carlsbad, CA, USA).

Cell culture. Human colorectal cell line colo 205 cell was purchased from the Food Industry Research and Development Institute (Hsinchu, Taiwan). Cells were plated onto 75 cm^2 tissue culture flasks in RPMI 1640 medium supplemented with 10% FBS, 100 Units/ml penicillin, 100 μg/ml streptomycin and 2 mM L-glutamine and grown at 37°C under a humidified 5% CO2 and 95% air at one atmosphere.

Cell morphological. Colo 205 cells (2.5×10^5 cells/well) were seeded onto 24-well cell culture plates at and then incubated with 20 μM of CTD for 24 h. Cells were directly examined and photographed under a contrast-phase microscope (12).

Cell viability. Colo 205 cells (2.5×10^4 cells/well) were seeded onto 96-well cell culture plates at and then incubated with 0, 10, 20 and 40 μM of CTD for 24, 48 and 72 h. After CTD incubation, 100 μl of MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) (5 μg/ml) was added to each well, and the plate was incubated at 37°C for 4 h. 100 μl of 0.04 N HCl/isopropanol was added and the absorbance at 570 nm was measured for each well. The cell survival ratio was expressed as % of control. All results were performed from three independent experiments (11).

Cell cycle analysis by flow cytometry. Colo 205 cells (2.5×10^5 cells/well) were seeded onto 24-well cell culture plates at and then incubated with 20 μM of CTD for 12, 24 and 48 h. The cells were harvested and washed by centrifugation. For apoptosis determination, cells were fixed by 70% ethanol in -20°C overnight and then re-suspended in PBS containing 40 μg/ml PI, 100 μg/ml RNase A and 0.1% Triton X-100 in dark room for 60 min. The cell cycle distribution and apoptotic cells (sub-G1 phase) were determined by flow cytometry (FACSCalibur™, Becton Dickinson, Franklin Lakes, NJ, USA). All results were performed from three independent experiments (15).

DAPI/TUNEL double staining. Colo 205 cells ($2x10^5$ cells/well) were seeded onto 24-well cell culture plates at and then incubated with 20 μM of CTD for 24 h. After

treatment, cells were harvested and immediately incubated with working strength terminal deoxynucleotidyl transferase (Tdt) enzyme (Invitrogen, Carlsbad, CA, USA) in 37°C for 1 h. The cells were immersed in stop/wash buffer and gently rinsed with PBS. FITC-labeled anti-digoxigenin conjugate was then applied to cells and incubated at 37°C for 30 min in the dark. The cells were washed in PBS then stained with DAPI dye and mounted with DABCO (Sigma-Aldrich). DAPI and TUNEL positive cells were visualized with a fluorescence microscope (13).

CDK1 kinase assay. Colo 205 cells ($1x10^7$ cells) were seeded onto 75T flask at and then incubated with 20 μM of CTD for 0, 12 and 24 h. Cells were suspended in a buffer containing, in a final volume of 0.2 ml, 50 mM Tris-HC1 (pH=7.5), 1 mM phenylmethylsulfonyl fluoride, 50 pg/ml leupeptin, 10 mM 2-mercaptoethanol, 1 mM $MgCl_2$, 2 mM EGTA, 0.5 mM dithiothreitol, 0.01% Brij35, 25 mM *b*-glycerophosphate, and 0.5 M NaCl. Cell suspensions were sonicated and centrifuged at 10,000×g for 30 min. To determine the CDK1 kinase assay condition using MV Peptide and determined by measuring OD 492 as described previously (17).

Annexin V assays. Colo 205 cells ($2.5x10^5$ cells/well) were seeded onto 24-well cell culture plates at and then incubated with 20 μM of CTD for 12 h. Cell was harvested the cells and incubated for 30 min with conjugated Annexin V-FITC (1 μg/mL) at room temperature in the dark, and analyzed by flow cytometry (Becton Dickinson FACSCalibur) (14).

Determination of mitochondrial membrane potential (△Ψm). Colo 205 cells ($2.5x10^5$ cells/well) were seeded onto 24-well cell culture plates at and then incubated with 20

μM of CTD for 0, 3, 6 and 12 h. The cells were harvested and washed for twice, then re-suspended in 500 ml of DiOC6 (4 mmol/L) and incubated at 37 ℃ for 30 min before analyzed by flow cytometry (Becton Dickinson FACSCalibur) (16).

Detection of reactive oxygen species (ROS) : Colo 205 cells ($2.5x10^5$ cells/well) were seeded onto 24-well cell culture plates at and then incubated with 20 μM of CTD for 0, 3, 6 and 12 h. The cells were harvested then washed for twice and re-suspended in 10 μM of the ROS dye DCFH-DA. Then the cells were incubated at 37˚C for 30 min and analyzed by flow cytometry (FACS Calibur™; Becton Dickinson)(11).

Caspase-3, -8 and -9 activities assay. Colo 205 cells (5.0×10^6 cells) were seeded in 6-well plates and treated with 20 μM of CTD for 24 h. Cells were harvested and lysed in 50 ml lysis buffer which contained 2 mM DTT for 10 min. Take the supernatant containing protein was incubated with caspase-3 substrate (Ac-DEVD-pNA), caspase-8 substrate (Ac-IETD-pNA) and caspase-9 substrate (Ac-LEHD-pNA) respectively in reaction buffer. And all samples were incubated in 96-well flat bottom plate at 37 ℃ for 1 h. The amounts of released pNA were measured at O.D. 405 nm with ELISA reader (11).

Western blot analysis. Colo 205 cells (2.5×10^7 cells) were seeded in 75-T flask and treated with 20 μM of CTD for 0, 6, 12, 18 and 24 h. The cells were harvested and washed with cold 1X PBS. The total proteins were collected. The protein concentration was measured by using a BCA assay kit (Pierce Biotechnology Inc., Rockford, IL, USA). Equal amounts of cell lysate were run on 10-12% SDS-polyacrylamide gel electrophoresis (SDS–PAGE) and electro-transferred to a

nitrocellulose membrane by using iBotTM Dry Blotting System (Invitrogen). The blot was soaked in blocking buffer (5% non-fat dry milk/0.05% Tween 20 in 20 mM TBS at pH 7.6) at room temperature for 1 h and then were incubated with anti-caspase-8, caspase-9, caspase-3, cyclin A, cyclin B, CDK1, CHK1, p21, cytochrome c, Bax, Bcl-2 and β-actin antibodies in blocking buffer at 4°C overnight. Membranes were washed three times with Tris-buffered saline/Tween 20 for 10 min and incubated with secondary horseradish peroxidase (HRP)-conjugated antibody. The blots were developed using a chemiluminescence (ECL) detection kit (Millipore, Billerica, MA, USA) followed by development on Kodak Bio-MAX MR film (Eastman Kodak, Rochester, NY, USA). All results were performed from three independent experiments (13).

Statistical analysis. Student's *t*-test was used to analyze the differences between the CTD-treated and control groups. All data were expressed as mean ± SD from at least three independent experiments. $***p < 0.001$ was indicative of significant difference.

Results

CTD reduces cell viability and induces morphological change in colo 205 cells.

We investigated the growth inhibition effect of CTD on colo 205 cells. Colo 205 cells were treated with different concentrations (0, 5, 10, 20 and 40 μM) of CTD, and cell viability was detected at 0, 24, 48 and 72 h by MTT assay. As shown in Figure 2A, the viability was significantly decreased in dose and time-dependent manners on CTD treated colo 205 cells groups. The IC_{50} at 24 h treatment is 20.53 μM in CTD treated colo 205 cells. Secondary, investigated the occurrence of morphological changes in colo 205 cell. It can be seen in Figure 2B that an apoptotic morphological changes,

including cell rounding and shrinkage after 24 h-incubation with 20 μM of CTD.

CTD induced G2/M arrest and apoptosis in colo 205 cells. We investigated the CTD induced colo 205 cell growth inhibition may through the cell cycle arrest and/or apoptotic mechanisms. Colo 205 cells were treated with 20 μM of CTD for 0, 12, 24, and 48 h, and cell cycle distribution was analyzed by flow cytometry. We found that CTD increased % of cells in G2/M group and sub-G1 growth (apoptosis) of colo 205 cells in a time-dependent manner (Fig. 3A). To confirm the CTD induced colo 205 cells apoptotic morphology, colo 205 cells were treated with 20 μM of CTD for 24 h then cells were harvested for determining the apoptosis by DAPI/TUNEL double staining. As shown in Figure 3B, DAPI/TUNEL double staining demonstrated that CTD induced DNA fragments and apoptosis.

CTD affected CDK 1 activity and the G2/M-associated protein levels in colo 205 cells. We investigated the CDK1 activity in G2/M phase on CTD treated colo 205 cells. As shown in Figure 4A, CTD caused a significant decrease in CDK1 activity at 12 and 24 h. We investigated the protein levels in G2/M phase of cell cycle. As shown in Figure 4B, CTD caused decrease in protein levels of Cyclin A, Cyclin B and CDK1 and caused an increase in protein levels of CHK1 and p21 in colo 205 cells. Our results suggest that CTD is able to decrease the CDK1 activity and to induce G2/M arrest in colo 205 cells

CTD induces phosphatidylserine (PS) translocation and Caspase-8/-9/-3 activities. To confirm the CTD induced colo 205 cells early apoptotic character, colo 205 cells were treated with 20 μM of CTD for 12 h then cells were harvested for determining by annexin V analysis. Treatment of colo 205 cells with CTD induced the

translocation of phosphatidylserine (PS) from inner side of the plasma membrane to the outer layer of the cell membrane by Annexin V analysis (Positive cells of CTD treated colo 205 cells: 44.22±2.68%; Fig. 5A). To investigate the mechanism of CTD induced apoptosis, we investigated the caspase-8/-9 and caspase-3 activities in CTD treated colo 205 cells. As shown in Figure 5B, CTD caused an increase of caspase-8, caspase-9 and caspase-3 activities in 24 h treatment. Our results suggest that the caspases activation may be involved in CTD-induced apoptotic cell death in colo 205 cells.

CTD-induced apoptosis was accompanied by the increase of ROS production and the loss of Mitochondrial membrane potential (MMP). We examined the effects of CTD on the ROS production and loss of mitochondrial membrane potential (MMP) by using the specific dye DCFH-DA and DiOC6. Our results shown in Figure 6, a remarkable increase of ROS production was evident after 3, 6 and 12 h in CTD treated colo 205 cells (Fig. 6A). A remarkable decrease in the mitochondrial membrane potential (MMP) was observed after 3, 6 and 12 h in CTD treated colo 205 cells (Fig. 6B). To further evaluate the role of ROS in CTD-induced growth inhibition in colo 205 cells, we pretreated with N-acetyl-L-cysteine (NAC), a ROS chelator, to block CTD-induced growth inhibition by MTT assay. As shown in Figure 6C, CTD-induced growth inhibition was significantly attenuated by N-acetyl-L-cysteine (NAC). Our results suggest that increase of ROS production and the loss of mitochondrial membrane potential (MMP) may be involved in the CTD induced apoptosis in colo 205 cells.

CTD affected the levels of apoptotic-associated proteins in colo 205 cells. We investigated the protein expression of Fas/CD95, caspase-8, caspase-3, cytochrome *c*,

caspase-9, Bax and Bcl-2 proteins levels by Western blotting analysis. As shown in Figure 7A, CTD increased the levels of Fas/CD95, cleavage caspase-3 and decreased the levels of pro-caspase-8. On the other hand, CTD also increased the levels of cytochrome *c*, Bax and decreased the levels of pro-caspase-9 and Bcl-2 (Fig. 7B). Our results suggested that the CTD-induced apoptotic through a death receptor-mediated and mitochondrial-mediated pathway.

Discussion

In this study, we investigated the anti-colorectal cancer activity of CTD on colo 205 cells; we focus on cell cycle arrest and cell apoptosis. Our results indicated CTD induced growth inhibition in colo 205 cells in dose- and time-dependent manners (Fig. 2A). Colo 205 cells treated with CTD showed G2/M phase arrest in cell cycle distribution (Fig. 3A). After treated with CTD in colo 205 cells, the CDK1 kinase activity was reduced, which broke G2/M progression. Our results also showed that the protein levels of CDK1, Cyclin A and Cyclin B were decreased after treated with CTD in colo 205 cells. Moreover, the level of p21 is up-regulation. It was also reported that the G2/M checkpoint plays an important role for DNA damage-induced apoptosis. The CDK1/Cyclin B complex is the major regulator leading the G2 to M phase progression (3, 19). Recently, it was reported that p21, a potent cyclin-dependent kinase inhibitor (CKI), is a key regulator, it can inhibits the CDK1 activity, strengthen the G2/M arrest of cell cycle (8, 18). It was also reported that CTD is a potent inhibitor of PP2A, which may block the APC activity. (Reference: Honkanen RE. Cantharidin, another natural toxin that inhibits the activity of serine／threonine protein phosphatases types 1 and 2A. FEBS Lett 1993; 330: 283–6.) CDK1 activity was inhibited by Cdh1, which is a co-activator of APC (Reference: Baker DJ, Dawlaty MM, Galardy P, van Deursen JM. Mitotic regulation of the

anaphase-promoting complex. Cell Mol Life Sci 2007; 64: 589–600.). Our results suggested that the treatment with CTD in colo 205 cells might be able to make confident APC through inhibition of PP2A and down-regulation of CDK1.

Activation of caspase is one of the major mechanisms that promote the cell apoptosis which response to death receptor signal (extrinsic or death-receptor pathway) and mitochondrial stress (intrinsic or mitochondrial pathway) (20-21). In this study, we first demonstrated that CTD reduced cell growth in colo 205 cells through induction of cell apoptosis. We provided strong evidence to approve that CTD induced apoptosis through the death receptor and mitochondrial apoptotic pathways in colo 205 cells. We showed in the present study that CTD: (1) decreased the percentage of viable cells by MTT assay (Fig.2A); (2) induced apoptotic morphological changes (Fig.2B); (3) induced DNA condensation and DNA fragmentation by DAPI/TUNAL staining (Fig. 3B); (4) induced translocation of phosphatidylserine (PS) from inner membrane to the outer membrane by Annexin V analysis (Fig.5A); (5) increased the protein levels of Fas/CD95, cleavage caspase-3, cytochrome *c*, Bax (Fig. 7); (6) activated caspase-8, caspase-9, and caspase-3 activities (Fig. 5B).

Previous studies have demonstrated that Bcl-2 and Bax locate in the mitochondrial outer-membrane and the Bcl-2/Bax ratio regulates the release of mitochondrial cytochrome *c* to cytosol. Our results showed that CTD promoted the expression of Bax (pro-apoptotic protein) and decreased the levels of Bcl-2 (anti-apoptotic protein) by western blotting (Fig. 7B). Furthermore, CTD increased the levels of cytochrome *c*, and decreased the levels of pro-caspase-9 (Fig. 7B). This is agreement with another report that CTD treatment led to dramatically decreased expression of Bcl-2 in human bladder carcinoma cell line. (References: Huan SK, Lee HH, Liu DZ, Wu CC and Wang CC: Cantharidininduced cytotoxicity and

cyclooxygenase 2 expression in human bladder carcinoma cell line. Toxicology 223: 136-143, 2006.) It was reported that CTD induced growth inhibition was not mitigated in pancreatic cancer cells with NAC (8). In our study, CTD induce apoptosis can increase the production of ROS on colo 205 cells (Fig. 6A), and CTD-induced growth inhibition was significantly attenuated by N-acetyl-L-cysteine (NAC). This is agreement with another report that the cytotoxic effect of cantharidin was believed to be related to oxidative stress (Reference: Rauh R, Kahl S, Boechzelt H, Bauer R, Kaina B, Efferth T. Molecular biology of cantharidin in cancer cells. Chin Med 2007; 2: 8.).

In conclusion, based on these observations, we have obtained convincing evidence that CTD efficiently inhibits the growth of human colorectal cancer cell lines colo 205 cells through CDK1 activity inhibition and caspase-dependent signal pathway as shown in Figure 8. Further investigation on both in vivo colorectal cancer models is necessary.

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Figure legends

Figure 1. The structure of cantharidin (CTD).

Figure 2. Effect of cantharidin on cell viability and morphology change of colo 205 cells. Colo 205 cells were treated with 0, 5, 10, 20 and 40 μM of cantharidin at 0, 24, 48 and 72 h; cells were harvested for determination of cell viability by using MTT assay (A). Cell were treated with 20 μM of cantharidin at 24 h then were examined and photographed for morphological changes by phase-contrast microscope (B). Data represent mean±SD of three experiments.

Figure 3. Effect of cantharidin on cell cycle distribution and apoptosis of colo 205 cells. Colo 205 cells were treated with 20 μM of cantharidin at 0, 12, 24 and 48 h then detected for the cell cycle distribution by flow cytometry (A). Colo 205 cells were treated with 20 μM of cantharidin at 24 h then detected for cell apoptosis by DAPI/TUNEL double staining, cells were examined and photographed under fluoresce microscopy (x400) as described in Materials and Methods (B).

Figure 4. Effect of cantharidin on CDK1 activity and G_2/M-associated protein levels of colo 205 cells. Colo 205 cells were treated with 20 μM of cantharidin at 0, 12 and 24 h, CDK1 kinase activity was measured in cellular extracts for the ability to phosphorylate MV Peptide, a CDK1 kinase specific substrate, according to the manual of Medical & Biological Laboratory's CDK1 kinase assay kit (A). Colo 205 cells were treated with 20 μM of cantharidin at 0, 6, 12, 18 and 24 h, then the total proteins were prepared then detected by Western blotting. Respectively, primary antibodies for Cyclin A, Cyclin B, CDK1, CHK1 and p21 were examined by Western blotting (B). ***$p<0.001$

Figure 5. Effect of cantharidin on phosphatidylserine (PS) translocation and Caspase-8, -9, -3 activities of colo 205 cells. Colo 205 cells were treated with 20 μM of cantharidin at 0 and 12 h then detected for the phosphatidylserine (PS) translocation by flow cytometry (A). Colo 205 cells were treated with 20 μM of cantharidin at 24 h. The total cell extracts were incubated with caspases-3, -9, and -8 specific substrates respectively (Ac-DEVD-pNA, Ac-LEHD-pNA, and Ac-IETD-pNA). The release of pNA was measured at 405 nm by a spectrophotometer (B). ***$p<0.001$

Figure 6. Effect of cantharidin on ROS production and loss of mitochondrial membrane potential (MMP) of colo 205 cells. Colo 205 cells were treated with 20 μM of cantharidin at 0, 3, 6 and 12 h then detected for the changes of ROS production (A) and loss of mitochondrial membrane potential (MMP) by DCFH-DA, DiOC6 dye, and the stained cells were determined by flow cytometry (B). Cells were pretreated with the N-acetyl-L-cysteine (NAC), a ROS chelator, for 1 hour and then treated with 20 μM of cantharidin at 24 h. Cells were harvested for determination of cell viability by using MTT assay (C). ***$p<0.001$

Figure 7. Effects of cantharidin on apoptotic relative protein levels of colo 205 cells. Western blotting analysis for Fas/CD95, caspase-8, caspase-3 (A) and cytochrome *c*, caspase-9, Bax, Bcl-2 protein levels (B) in cantharidin examined colo 205 cells. For Western blotting analysis, total protein extracts were analyzed by immunoblotting.

Figure 8. The proposed model of cantharidin-mediated G2/M arrest and apoptosis in colo 205 cells.

Figure 1

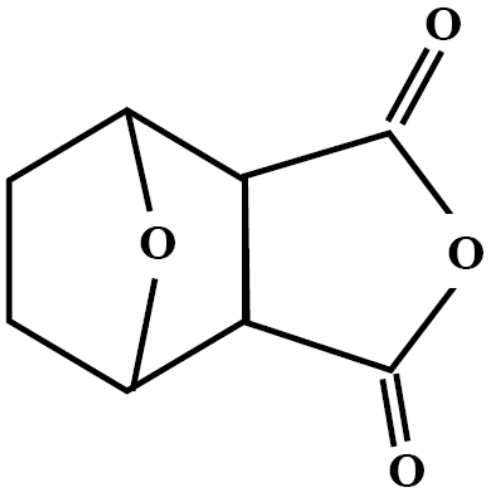
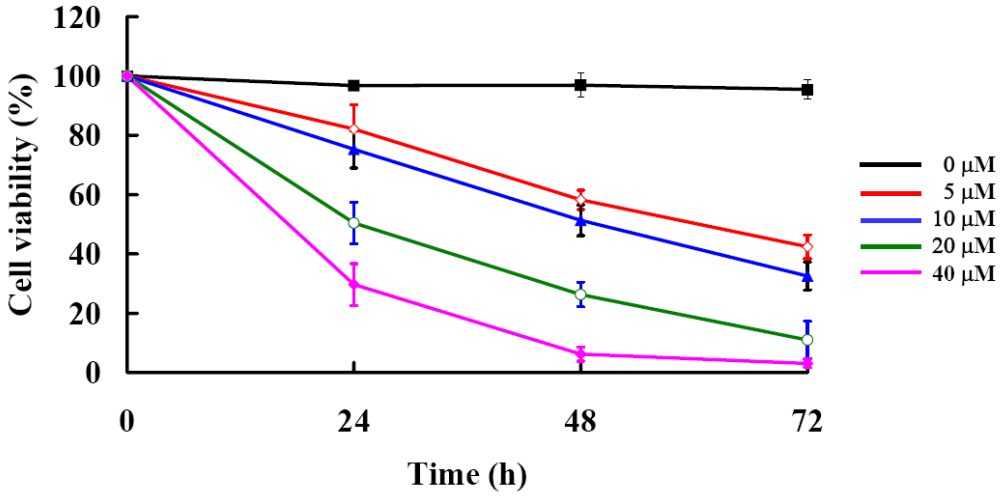
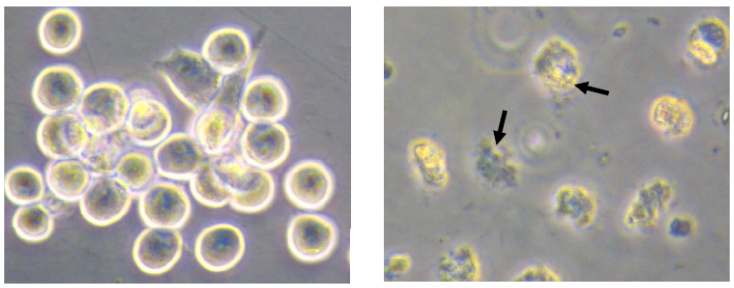


Figure 2

A



B



Control

Cantharidin (20 μM)

Figure 3

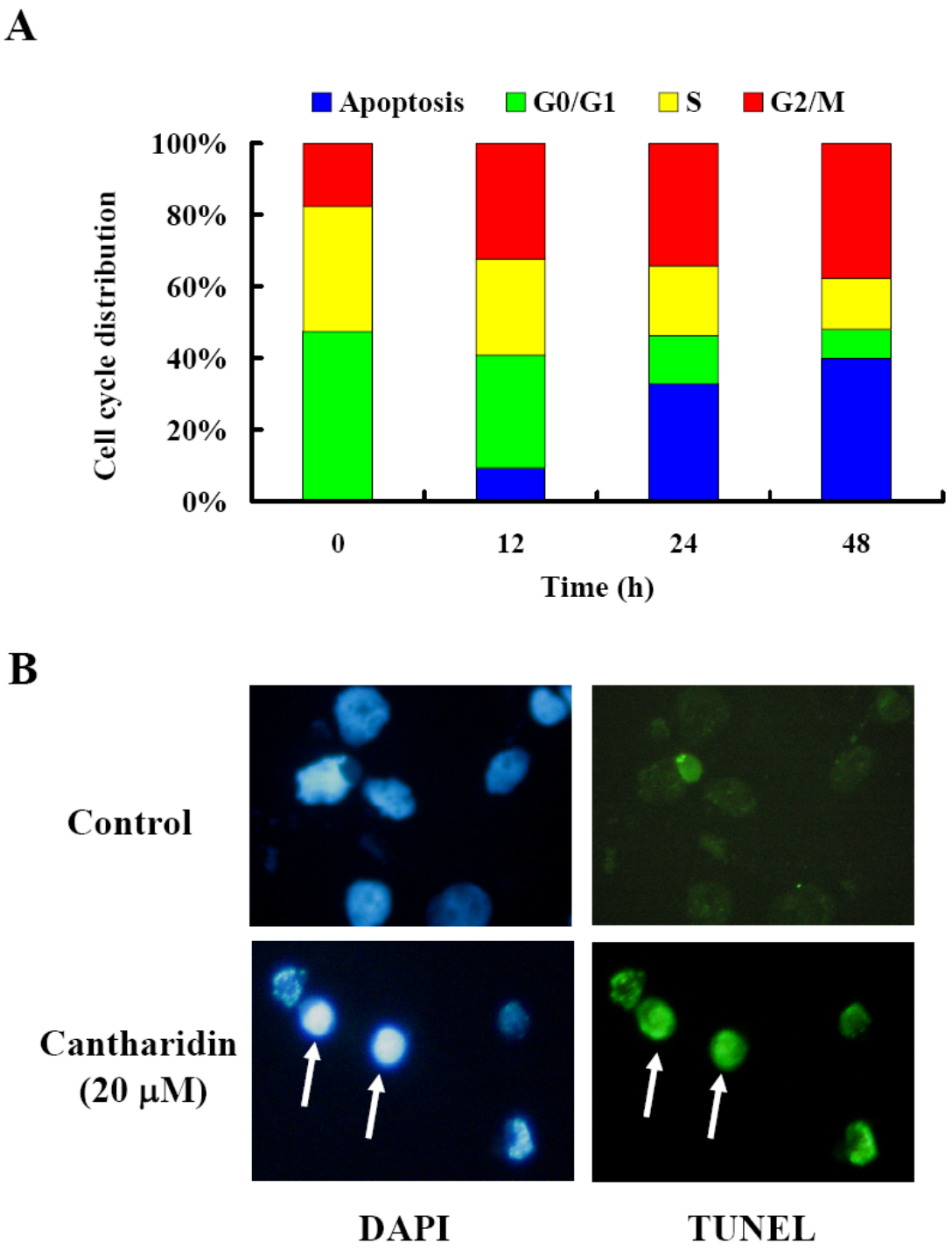
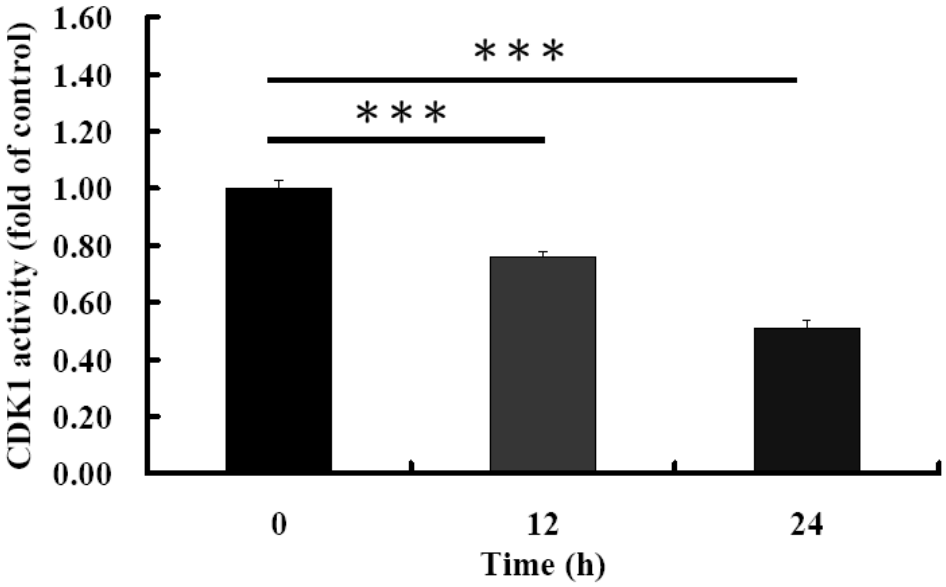

A
Apoptosis
G0/G1
S
G2/M
100%
80%
60%
40%
20%
0%
Cell cycle distribution
0
12
24
48
Time (h)
B
Control
Cantharidin
(20 μM)
DAPI
TUNEL

Figure 4

A


CDK1 activity (fold of control)
1.60
1.40
1.20
1.00
0.80
0.60
0.40
0.20
0.00
***
***
0
12
24
Time (h)

B

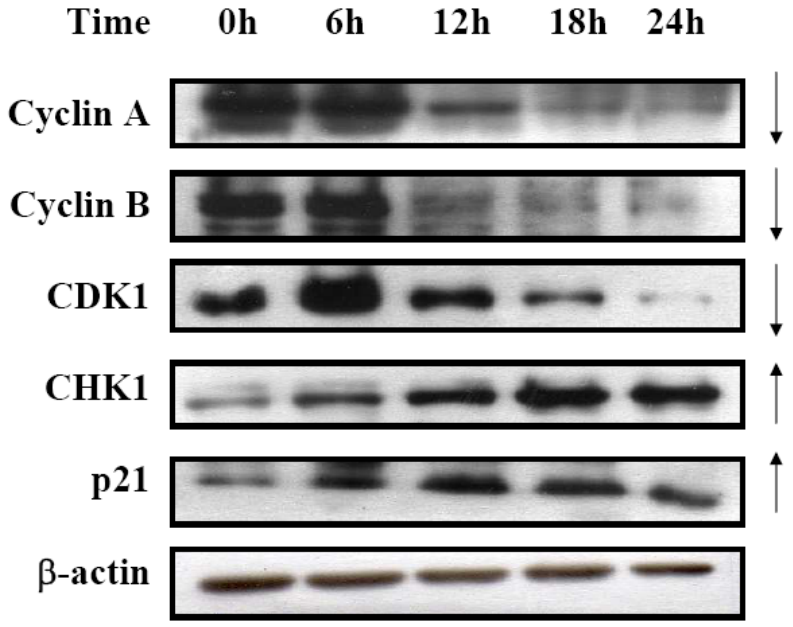
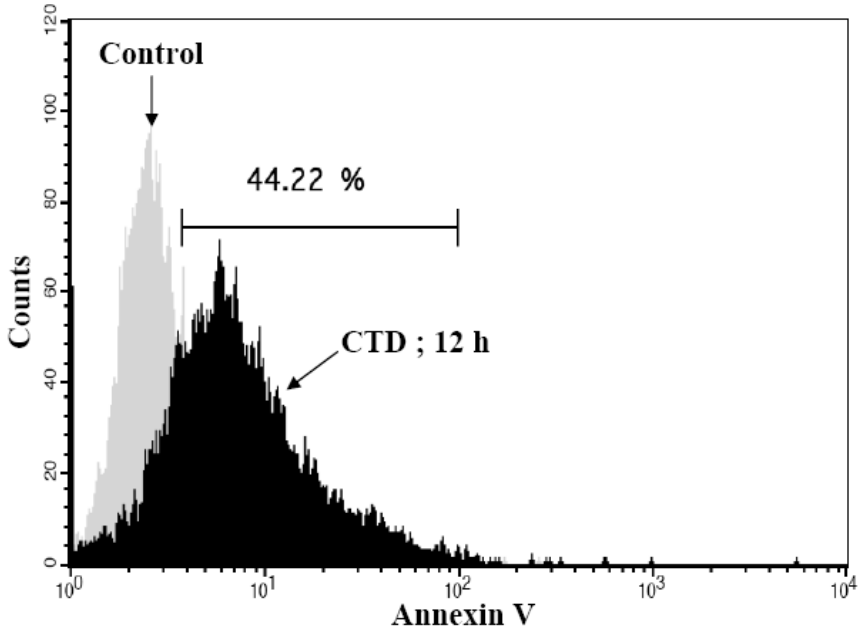

Time
0h
6h
12h
18h
24h
Cyclin A
Cyclin B
CDK1
CHK1
p21
β-actin

Figure 5

A


Control
44.22 %
CTD ; 12 h
Counts
Annexin V
0
20
40
60
80
100
120
10^0
10^1
10^2
10^3
10^4

B

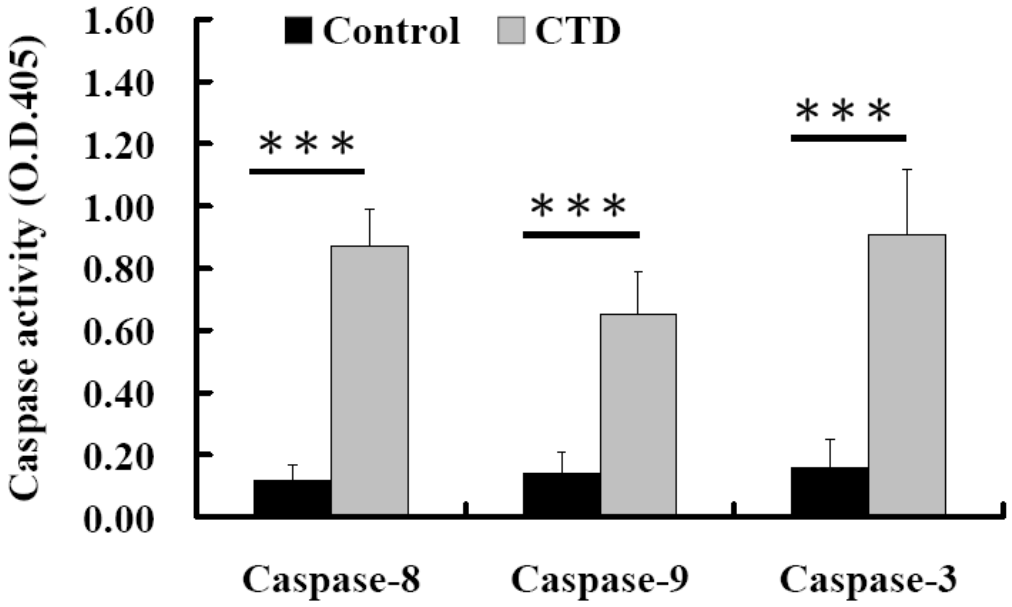

Control
CTD
Caspase activity (O.D.405)
0.00
0.20
0.40
0.60
0.80
1.00
1.20
1.40
1.60
***
***
***
Caspase-8
Caspase-9
Caspase-3

Figure 6

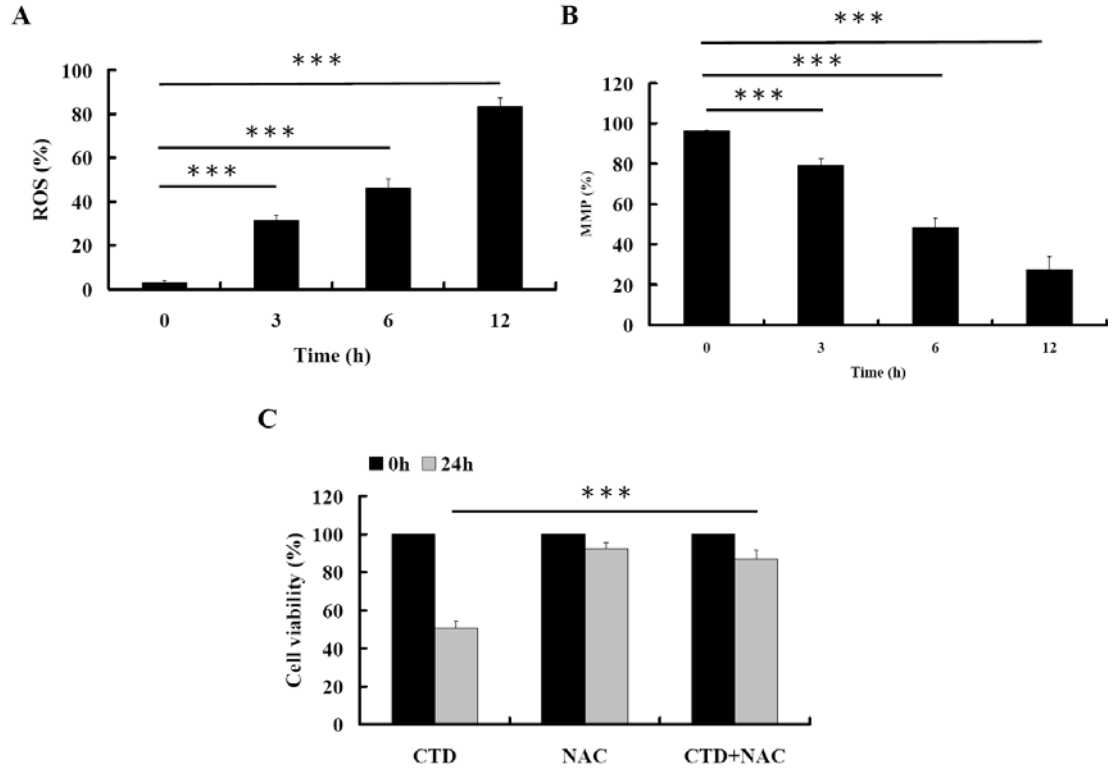

A
ROS (%)
100
80
60
40
20
0
***
***
***
0
3
6
12
Time (h)
B
MMP (%)
120
100
80
60
40
20
0
***
***
***
0
3
6
12
Time (h)
C
0h
24h
Cell viability (%)
120
100
80
60
40
20
0
***
CTD
NAC
CTD+NAC

Figure 7

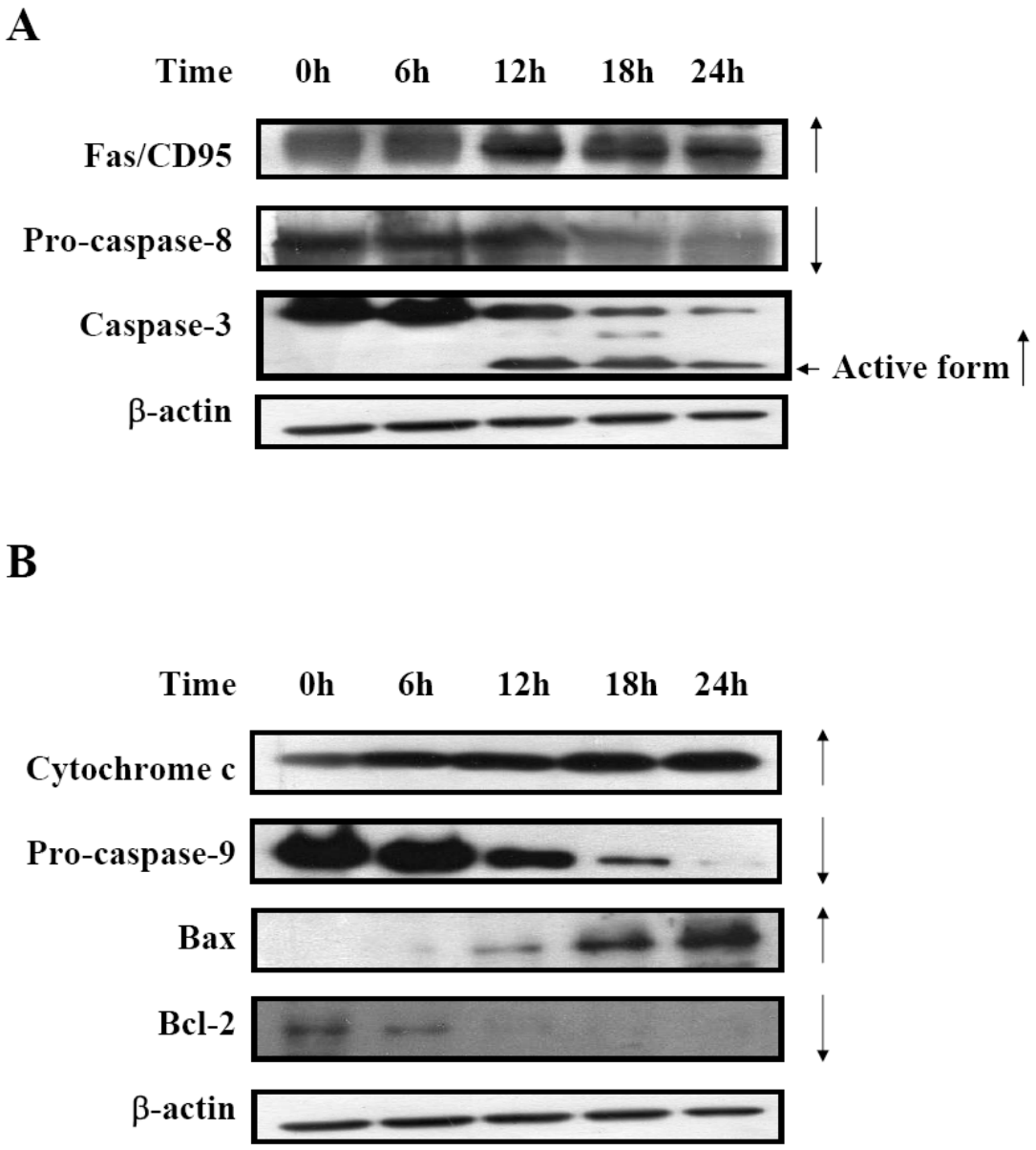
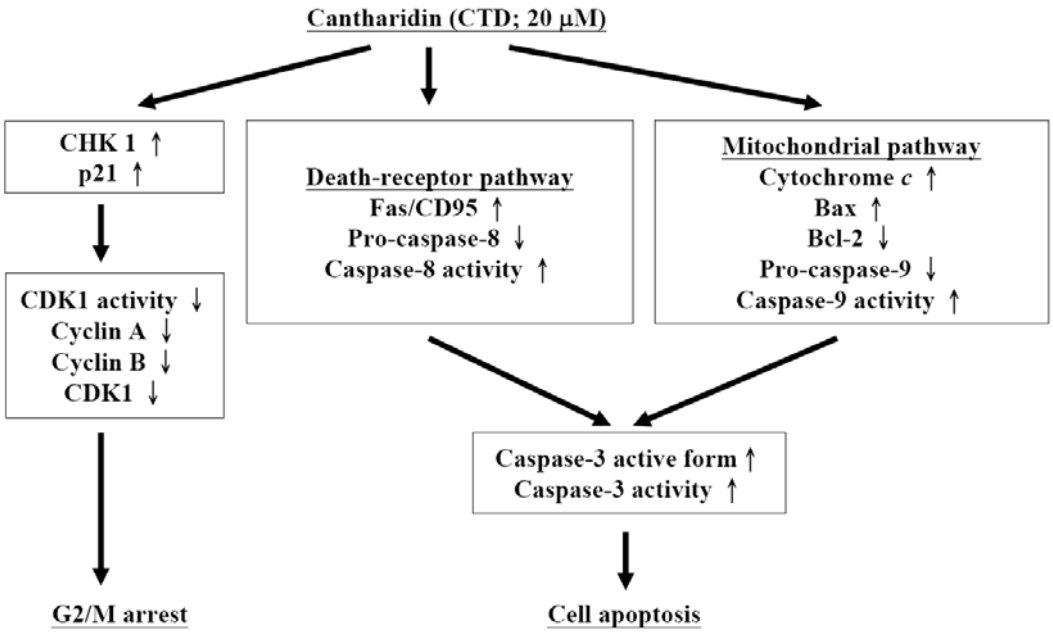

A
Time
0h
6h
12h
18h
24h
Fas/CD95
Pro-caspase-8
Caspase-3
Active form
β-actin
B
Time
0h
6h
12h
18h
24h
Cytochrome c
Pro-caspase-9
Bax
Bcl-2
β-actin

Figure 8


Cantharidin (CTD; 20 μM)
CHK 1 ↑
p21 ↑
CDK1 activity ↓
Cyclin A ↓
Cyclin B ↓
CDK1 ↓
G2/M arrest
Death-receptor pathway
Fas/CD95 ↑
Pro-caspase-8 ↓
Caspase-8 activity ↑
Mitochondrial pathway
Cytochrome c ↑
Bax ↑
Bcl-2 ↓
Pro-caspase-9 ↓
Caspase-9 activity ↑
Caspase-3 active form ↑
Caspase-3 activity ↑
Cell apoptosis