Norcantharidin-induced Apoptosis of AGS Human Gastric Cancer Cells Through Reactive Oxygen Species Production, and Caspase- and Mitochondria-dependent Signaling Pathways

LI-CHENG ZHENG^{1#}, MEI-DUE YANG^{2,3,4#}, CHAO-LIN KUO⁵, CHIA-HSIN LIN⁵, MING-JEN FAN⁶,
YU-CHENG CHOU⁷, HSU-FENG LU⁸, WEN-WEN HUANG¹, SHU-FEN PENG^{1*} and JING-GUNG CHUNG^{1,6*}

Departments of ¹Biological Science and Technology, and ⁵Chinese Pharmaceutical Sciences and Chinese Medicine Resources, China Medical University, Taichung, Taiwan, R.O.C.;

Departments of ²Surgery and ³Clinical Nutrition, and ⁴Terry Fox Cancer Research Laboratory, China Medical University Hospital, Taichung, Taiwan, R.O.C.;

⁶Department of Biotechnology, Asia University, Taichung, Taiwan, R.O.C.;

⁷Division of Neurosurgical Oncology, Neurological Institute, Taichung Veterans General Hospital, Taichung, Taiwan, R.O.C.;

⁸Department of Clinical Pathology, Cheng Hsin General Hospital, Taipei, Taiwan, R.O.C.

Abstract. *Norcantharidin (NCTD) was purified from mylabris, the dried body of the Chinese blister beetle. NCTD has been shown to exhibit anticancer activities in many human cancer cell lines, but there are no reports to show whether it induces apoptosis of human gastric cancer cells. Therefore, in the present study, we investigated NCTD-induced cell death and associated protein expression in human gastric cancer AGS cells in vitro. Cell morphological changes, viability and cell-cycle distribution were examined and analyzed by phase-contrast microscopy and flow cytometric assays. Flow cytometry was also used to measure the levels of reactive oxygen species (ROS), Ca^{2+} , mitochondrial membrane potential (Ψ_m) and activity of caspases. The results indicated that NCTD induced cell morphological changes, reduced total viable cell number and induced G_0/G_1 phase arrest. NCTD also increased ROS production and reduced the Ψ_m and increased caspase-9*

activity in AGS cells. Western blotting also found that NCTD increased the pro-apoptotic proteins such as BCL2-associated X protein (BAX) and BH3 interacting-domain death agonist (BID) and increased the release of cytochrome c, apoptosis inducing factor (AIF) and endonuclease G (Endo G) release from mitochondria in AGS cells. NCTD also significantly increased the expression of active forms of caspase-3 and -8 and -9 and reduced the expression of caspase-4 and -12 in AGS cells. Based on these observations, we suggest that NCTD-induced apoptotic cell death may be through mitochondria- and caspase-dependent pathways.

Gastric cancer is the seventh most common cancer in Taiwan and 10 individuals per 100,000 die annually from gastric cancer based on the 2014 report from the Department of Health (Taiwan) (1). Risk factors include genetic factors, lifestyle and environmental toxic chemicals (2, 3). Currently, primary surgery, radiation therapy, chemotherapy or the combination of chemo- and radiotherapy are the major treatment options for patients with gastric cancer.

Apoptosis, a type of programmed cell death, plays a critical role in a wide variety of physiological processes during fetal development and in adult tissues (4). Cell apoptotic features include cell shrinkage, nuclear collapse, membrane blebbing, and DNA fragmentation (5, 6). Thus, this process has been the most frequently taken as a major pathway for chemotherapeutics to combat cancer cells. It is well documented that cell apoptosis is mediated through extrinsically and intrinsically mediated pathways. The extrinsically mediated pathway is triggered through activation of cell surface ligand-gated death receptors and the intrinsically

#*These Authors contributed equally to this work.

Correspondence to: Jing-Gung Chung, Ph. D., Department of Biological Science and Technology, China Medical University, No 91, Hsueh-Shih Road, Taichung, Taiwan, R.O.C. Tel: +886 422053366 ext 8000, Fax: +886 422053764, e-mail: jgchung@mail.cmu.edu.tw and Shu-Fen Peng, Department of Biological Science and Technology, China Medical University, No 91, Hsueh-Shih Road, Taichung, Taiwan, R.O.C. Tel: +886 422053366 ext 2506, Fax: +886 422071507, e-mail: sfpeng@mail.cmu.edu.tw

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mediated pathway involves the mitochondria, which are disrupted by cell stress (6) followed by cytochrome *c* release thereby inducing apoptosome formation and activating caspases causing cell apoptosis (7, 8).

One strategy for anticancer function of drugs is to trigger tumor cell apoptosis in patients (9). Numerous experiments have been undertaken to find novel compound from natural products to achieve this in treating patients with gastric cancer.

Norcantharidin (NCTD) is a bioactive compound and low-toxicity analog of the active anticancer compound cantharidin, purified from mylabris, the dried body of the Chinese blister beetle (10). Much evidence has demonstrated that NCTD exerts antitumor activities against hepatoma (11), medulloblastoma (12), bladder carcinoma (13), myeloid leukemia (14), melanoma (15) and gallbladder carcinoma (16) cells. NCTD induced DU145 cell apoptosis through reactive oxygen species (ROS)-mediated mitochondrial dysfunction and ATP depletion (10) and may exert its anticancer activity through the suppression of the RAF kinase/mitogen-activated protein kinase/extracellular signal-regulated kinases (RAF/MEK/ERK) pathway (17). NCTD has anti-vasculogenic mimicry activity against human gallbladder cancer possibly *via* blocking the ephrin type A receptor 2/focal adhesion kinase/paxillin signaling pathway (18). NCTD inhibits lymphangiogenesis by down-regulating the expression of vascular endothelial growth factor-C (VEGFC) and VEGFD (19). Recently, it was indicated that the induction of apoptosis of human hepatocellular carcinoma HepG2 and SMMC-7721 cells with treatment of ABT-737 (an antagonist of B-cell lymphoma 2, BCL2) combined with NCTD for 48 h was greater than that of both ABT-737 and NCTD alone (20).

Although numerous studies have shown that NCTD induced cell death in many human cancer cell lines, the anticancer activity of NCTD on human gastric cancer cells *in vitro*, and its underlying mechanisms, have not been fully investigated. Therefore, in the present study, we investigated the efficacy of NCTD on AGS gastric cancer cells and attempted to elucidate detailed mechanisms of anticancer activity.

Materials and Methods

Chemicals and reagents. NCTD of 99% purity, 6-diamidine-2-phenylindole (DAPI), dimethyl sulfoxide (DMSO), propidium iodide (PI) and trypsin-EDTA were obtained from Sigma Chemical Co. (St. Louis, MO, USA). RPMI-1640 medium, fetal bovine serum (FBS), L-glutamine and penicillin-streptomycin were purchased from GIBCO®/Invitrogen Life Technologies (Carlsbad, CA, USA). Primary antibody against caspase-3, -7, -8, -9, p21^{CLP1/WAF1} (p21), p27^{KIP1} (p27), apoptosis inducing factor (AIF), Bcl2-associated X protein (BAX), BH3 interacting-domain death agonist (BID) and BCL2 were purchased from Santa Cruz Biotechnology, Inc. (Dallas, TX, USA) and those against poly (ADP-ribose) polymerase (PARP), cytochrome

c, endonuclease G (Endo G), second mitochondrial-derived activator of caspase (SMAC), X chromosome-linked inhibitor of apoptosis protein (XIAP), p53, Fas receptor (FAS), Fas ligand (FASL), death receptor 4 (DR4), death receptor 5 (DR5), tumor necrosis factor α (TNF α), TNF-related apoptosis-inducing ligand (TRAIL), glucose-regulated protein 78 (GRP78), cyclin D, cyclin-dependent kinase 6 (CDK6), caspase-4, caspase-12, calpain-1 and peroxidase-conjugated secondary antibodies were purchased from Cell Signaling Technology, Inc. (Beverly, MA, USA). NCTD was dissolved in DMSO. Cell culture grade DMSO was used for vehicle at 0.1%.

Cell culture. The human gastric cancer AGS cell line was obtained from the Food Industry Research and Development Institute (Hsinchu, Taiwan, ROC). AGS cells were grown in RPMI-1640 medium supplemented with 10% heat inactivated FBS and antibiotics (100 units/ml penicillin, 100 μ g/ml streptomycin, and 2 mM glutamine) was routinely grown at 37°C and 5% (v/v) CO₂ (21).

Cell morphology and viability measurements. AGS cells (1 $\times$ 10⁵ cells/well) were seeded in 12-well plates with RPMI-1640 for 24 h. NCTD at final concentrations of 10, 15, 20, 25 and 30 μ M, or 0.1% DMSO as a vehicle control was added to each well and cells were cultured for 48 h. Cells were then examined and photographed under phase-contrast microscopy at $\times$ 200 for examination of cell morphological changes. Cells were harvested, counted and stained with PI (5 μ g/ml) for total viable cell number measured by flow cytometry (BD Biosciences, FACSCalibur, San Jose, CA, USA) as previously described (22, 23).

Cell cycle and sub-G₁ phase (apoptotic cell death) assays. Sub-G₁ hypodiploid cells and cell-cycle distribution were quantified by flow cytometry as previously described (21). AGS cells (1 $\times$ 10⁵ cells/well) in 12-well plates were incubated with NCTD (10, 15, 20, 25 and 30 μ M) for 48 h and were harvested, washed and fixed in 70% ethanol for 30 min at 37°C in the dark with a solution containing 50 mg/ml PI and 50 μ g/ml RNase A. Cells were then analyzed by FACSCalibur flow cytometer (Becton Dickinson) and results were determined by CellQuest and ModFit computer programs as described previously (21).

Annexin V-FITC labeling. AGS cell apoptosis was measured by using the Annexin V/PI kit (BD Pharmingen, San Diego, CA, USA) as described previously (21). AGS cells (1 $\times$ 10⁵ cells/well) in 12-well plates were exposed to 30 μ M NCTD for 24 and 48 h. Cells were washed twice with PBS solution and then resuspended cells in binding buffer [0.01 M Hepes/NaOH (pH 7.4), 0.14 M NaCl, 2.5 mM CaCl₂] and the following cells were stained with annexin V/PI (100 μ l binding buffer, 5 μ l annexin-V and 5 μ l PI) for 15 min at room temperature in the dark, and then were measured by flow cytometry (21).

Nuclear staining with 4,6-diamidino-2-phenylindole (DAPI). DAPI staining was carried out as previously described (21). AGS cells (1 $\times$ 10⁵ cells/well) were placed in 12-well plates and cells were incubated with NCTD (10, 15, 20, 25 and 30 μ M) for 24 and 48 h. Cells were fixed in 4% formaldehyde in PBS for 20 min at room temperature and were washed with PBS and stained with DAPI solution (2 μ g/ml) (Santa Cruz Biotechnology, Inc., Dallas, TX, USA) at room temperature in the dark. Nuclear morphology was examined and photographed using a fluorescence microscope as described previously (21).

Figure 1. Norcantharidin (NCTD) induced cell morphological changes and reduced the percentage of viable AGS cells. Cells were treated with NCTD for 48 h and the cells were examined and photographed for cell morphological changes by contrast-phase microscopy at $\times 200$ (A) and were collected for measurement of total number of viable cells (B) by flow cytometry as described in the Materials and Methods Section. The results are shown as the mean $\pm$ SD ($n=3$). * $p<0.05$, Significantly different from the control as analyzed by Student's *t*-test.

Measurements of ROS, intracellular Ca^{2+} and mitochondrial membrane potential (Ψ_m). Flow cytometry was used for these experiments. AGS cells (1×10^5 cells/well) in 12-well plates were incubated with NCTD (30 μ M) for different time periods. At the end of incubation, cells were isolated and resuspended with 500 μ l of 2',7'-dichlorodihydrofluorescein diacetate (DCFH-DA) (10 μ M) for ROS measurement, or with 500 μ l of 3,3'-dihexyloxycarbocyanine iodide (DiOC₆) (4 μ mol/l) for Ψ_m , or 500 μ l of Fluo-3/acetoxymethyl ester (Fluo-3/AM) (2.5 μ g/ml) for intracellular Ca^{2+} determination in the dark for 30 min and then cells were analyzed by flow cytometry as described previously (24, 25).

Measurements of caspase-9 activity. The activity of caspase-9 was measured by flow cytometric assay (21). AGS cells (1×10^5 cells/well) in 12-well plate were pretreated or with pan-caspase inhibitor (Z-VAD-FMK) for 3 h and with/without NCTD (30 μ M) for 0, 6, 12, 24 and 48 h. After incubation, cells were collected, washed and re-suspended in 50 μ l of 10 μ M substrate solution containing CaspaLux9-M1D2 for caspase-9 activity measurement

before being incubated at 37°C for 60 min. Cell samples were further analyzed by flow cytometry for caspase-9 activity and total viable cells as described previously (26).

Western blot analysis. AGS cells (1.5×10^6 cells/dish) were placed in 10-cm dish for 24 h then were incubated with NCTD (30 μ M) for 0, 6, 12, 24 and 48 h. Cells were collected, lysed and total protein was determined by Bio-Rad protein assay kit (Bio-Rad Hercules, CA, USA) as described previously (21). Thirty micrograms of total cellular proteins were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (12% SDS-PAGE), transferred onto a polyvinylidene difluoride membranes (Millipore, Billerica, MA, USA) which were subsequently hybridized with the primary antibodies (1:1,000 dilution) and β -actin at 4°C overnight. After washing with PBS-Tween 20 buffer, the membrane was incubated with secondary antibody (1:5,000 dilution) anti-mouse IgG (Santa Cruz Biotechnology) for 1 h at room temperature. Protein bands were visualized by the enhanced chemiluminescence using ECL detection (GE Healthcare Bio-Sciences, Pittsburgh, PA, USA) (27, 28).

Figure 2. Norcantharidin (NCTD) induced G_0/G_1 arrest and sub- G_1 phase in AGS cells. AGS cells were treated with different concentrations of NCTD for 48 h or were treated with 30 μ M for different time periods. Cells were harvested for cell-cycle distribution by flow cytometric assay (A) and quantified by FACSCalibur flow cytometry (B). Protein expression was measured by western blotting as described in the Materials and Methods (C). The results are shown as the mean $\pm$ SD ($n=3$). * $p<0.05$, Significantly different from the control as analyzed by the Student's t -test. CDK6: Cyclin-dependent kinase 6.

Statistical analysis. The results are presented as the mean $\pm$ standard deviation from three independent experiments. Significant differences among the groups were determined using the unpaired Student's t -test. The differences were considered statistically significant at $p<0.05$.

Results

NCTD induced cell morphological changes and reduced the cell viability of AGS cells. Cell morphology and viability

Figure 3. Norcantharidin (NCTD) induced apoptotic cell death of AGS cells. Cells were treated with 0 or 30 μ M of NCTD for 24 and 48 h then the cells were double stained with annexin V/propidium iodide (PI) and were analyzed for apoptotic cell death (A) and quantified by flow cytometric assay (B) as described in the Materials and Methods section. The results are shown as the mean $\pm$ SD ($n=3$). * $p<0.05$, Significantly different from the control as analyzed by the Student's *t*-test.

were performed by phase-contrast microscopy and flow cytometric assay, respectively. The results indicate that NCTD induced cell morphological changes (Figure 1A) and reduced total viable cell number (viability) of AGS cells dose-dependently (Figure 1B), with a half maximal inhibitory concentration (IC_{50}) of 30 μ M at 48 h treatment.

NCTD induced G_0/G_1 arrest and sub- G_1 phase in AGS cells. In order to understand whether NCTD reduced total viable cell number through cell-cycle arrest and cell apoptosis in AGS cells, AGS cells were treated with 0, 10, 15, 20, 25, 30 μ M of NCTD for 48 h and were harvested for cell cycle distribution and sub- G_1 phase assays. The results indicate that NCTD induced G_0/G_1 phase arrest (Figure 2A) and accumulation of cells in sub- G_1 phase (apoptosis) (Figure 2B) and these effects were somewhat dose-dependent. For further investigating whether NCTD induced G_0/G_1 phase arrest through the effects of cell-cycle check point proteins, cells were harvested for western blotting. NCTD increased the protein expression of p27 at all examined time but p21 was increased from 6-24 h treatment. However, cyclin D and CDK6 decreased after 48 h treatment.

NCTD induced apoptosis of AGS cells. For further understanding of whether the growth-inhibitory effect of NCTD is associated with cell apoptosis, annexin V/PI double staining of AGS cells by flow cytometric analyses were used. The number of apoptotic cells increased in a time-dependent manner after incubation with 30 μ M NCTD compared to the control group (Figure 3). These results indicate that inhibition of cell growth by NCTD was due to induction of cell apoptosis.

NCTD induce nuclear condensation in AGS cells. AGS cells were treated with 0, 10, 15, 20, 25 and 30 μ M of NCTD for 24 and 48 h and then were stained with DAPI, examined and photographed by fluorescence microscopy, as shown in Figure 4A-C. Figure 4C indicates that longer treatment increased fluorescence of AGS cells when compared to control cells, as did increasing concentrations of NCTD. Increasing fluorescence reflects the increasing presence of nicked DNA and nuclear chromatin condensation in cells and these effects were also revealed to occur in a dose-dependent manner.

NCTD induced ROS and Ca^{2+} production and reduced the mitochondrial membrane potential (Ψ_m) in AGS cells. AGS

Figure 4. Norcantharidin (NCTD) induced nuclear chromatin condensation in AGS cells. Cells were treated with NCTD for 24 and 48 h then were stained with 4',6-diamidino-2-phenylindole (DAPI) as described in the Materials and Methods section. The results are shown as the mean $\pm$ SD (n=3). *p<0.05, Significantly different from the control as analyzed by Student's t-test.

A

B

C

Figure 5. Norcantharidin (NCTD) induced reactive oxygen species (ROS) and Ca^{2+} production and reduced the mitochondrial membrane potential (Ψ_m) in AGS cells. Cells (1×10^5 cells/well) were treated with $30 \mu\text{M}$ of NCTD for different time periods. Cells were isolated and assayed for the production of ROS (A), $\Delta\Psi_m$ measurement (B) and Ca^{2+} (C) as described in the Materials and Methods section. The results are shown as the mean $\pm$ SD ($n=3$). * $p<0.05$, Significantly different from the control as analyzed by the Student's *t*-test.

Figure 6. Measurement of caspase-9 activity. A: AGS cells (2×10^5 cells/well) in 12-well plates were pretreated or not with pan-caspase inhibitor (Z-VAD-FMK) and then treated with/without 30 μ M of norcantharidin (NCTD) for 24 h. Cell viability was analyzed by flow cytometry as described in the Materials and Methods section. B: Cells were treated with 30 μ M of NCTD for 0, 6, 12, 24 and 48 h then resuspended in 50 μ l of 10 μ M substrate solution containing Caspase-9-MID2 for caspase-9 activity measurement by flow cytometry as described in the Materials and Methods Section.

cells were treated with 30 μ M of NCTD for different time periods and were harvested and analyzed by flow cytometric assay, as shown in Figure 5. NCTD increased ROS production from 3-12 h treatment (Figure 5A). NCTD reduced the mitochondrial membrane potential from 6 h up to 48 h treatment (Figure 5B), however, it did not significantly affect the level of Ca^{2+} production from 6-48 h treatment (Figure 5C) when compared to the control group. These results indicated that ROS, and $\Delta\Psi_m$ are involved in NCTD-induced apoptosis of AGS cells *in vitro*.

NCTD increased the activity of caspase-9 in AGS cells. In order to investigate whether NCTD induced cell apoptosis involves caspase in AGS cells, cells were pretreated with pan-caspase inhibitor (Z-VAD-FMK) for 3 h and then treated or not with 30 μ M of NCTD. Treatment with NCTD and pan-caspase inhibitor increased the number of viable AGS cells compared to those only treated with NCTD (Figure 6A). Treatment of AGS cells with 30 μ M of NCTD for 6, 12, 24 and 48 h led to increased caspase-9 activity as shown in Figure 7A. These results indicate that NCTD induces AGS cell apoptosis through the activation of caspase-9 *in vitro*.

NCTD altered the expression of apoptosis-associated proteins in AGS cells. To investigate whether NCTD induced cell apoptosis involved changes in expression of apoptosis-associated proteins in AGS cells, cells were treated with 30 μ M of NCTD for 6, 12, 24 and 48 h and proteins were examined by western blotting, as shown in Figure 7. The results showed that NCTD significantly increased the expression of active form of caspase-3, -9 and -8 (Figure 7A), BAX, BID, BCL2, PARP, AIF, Endo G, and cytochrome c (Figure 7B), p53, XIAP and SMAC (Figure 7C), DR4, DR5, TRAIL and TNF α (Figure 7D) at all examined treatment times. However, caspase-12 and -4 (Figure 7E) were increased at 6-24 h treatment of NCTD. NCTD reduced the expression of caspase-7 (Figure 7A), Fas and FasL (Figure 7D), calpain-1 and GRP78 in AGS cells (Figure 7E).

Discussion

Numerous studies have demonstrated that NCTD induces cytotoxic effects on many human cancer cell lines through cell-cycle arrest and apoptosis, however, there is no available information to show that NCTD affects human gastric cancer AGS cells. In the present study, we investigated the cytotoxic effects of NCTD on human gastric cancer AGS cells *in vitro*. Herein, we found that NCTD induced G_0/G_1 phase arrest in AGS cells, which is in agreement with a report which showed that NCTD induced G_0/G_1 phase arrest at 25 μ M. However, at high doses (50 μ M), it led to increase G_2/M phase arrest (29). The mechanisms of NCTD-mediated regulation of cell cycle-related protein expression were investigated. Short

Figure 7. Norcantharidin (NCTD) affects expression of apoptosis-associated proteins in AGS cells. Cells were treated with 30 μ M of NCTD for 0, 6, 12, 24 and 48 h. Cells were then collected and total proteins were quantitated and apoptosis-associated proteins were examined by western blotting as described in the Materials and Methods section. A: Caspase-3, -8, -9 and -7; B: poly (ADP-ribose) polymerase (PARP), BCL2: B-cell lymphoma 2, BCL2-associated X protein (BAX), BH3 interacting-domain death agonist (BID), apoptosis-inducing factor (AIF), cytochrome c and endonuclease G (Endo G); C: second mitochondrial-derived activator of caspase (SMAC), X chromosome-linked inhibitor of apoptosis protein (XIAP) and p53; D: Fas, FasL, death receptor 4 (DR4), DR5, tumor necrosis factor α (TNF α) and TNF-related apoptosis-inducing ligand (TRAIL); E: caspase-12 and -4, calpain-1 and glucose-regulated protein 78 (GRP78).

Figure 8. The proposed signaling pathways of norcantharidin (NCTD)-induced apoptosis in AGS cells. ROS: Reactive oxygen species; PARP: poly (ADP-ribose) polymerase; AIF: apoptosis-inducing factor; Endo G: endonuclease G; SMAC: second mitochondrial-derived activator of caspase; XIAP: X chromosome-linked inhibitor of apoptosis protein; DR/DR5: death receptor 4/5; TNF α : tumor necrosis factor α ; TRAIL: TNF-related apoptosis-inducing ligand; GRP78: glucose-regulated protein 78; CDK6: cyclin-dependent kinase 6; BAX: BCL2-associated X protein; BID: BH3 interacting-domain death agonist; MMP: matrix metalloproteinase.

NCTD treatment led to increased p21 and p27 but inhibited expression of cyclin D and CDK6 in AGS cells. Other studies have shown that the functional property of the phosphorylation of p21^{CIP1/WAF1} upon NCTD treatment is still unclear (30). The possible role of p21^{CIP1/WAF1} in G₀/G₁ phase in AGS cells needs further investigation.

Sub-G₁ phase population of AGS cells was observed after exposed to different concentrations of NCTD. The sub-G₁ phase is recognized to be one of the hallmarks of cell apoptosis (31, 32). Annexin V/PI double staining confirmed here that NCTD induced cell apoptosis of AGS cells; NCTD has been shown to induce apoptosis of multiple types of cancer cells (10, 15, 33, 34). We also used DAPI staining to show NCTD induced chromatin condensation to show NCTD induce DNA damaged, in agreement with a report on TSGH 8301 human urinary bladder carcinoma cells (13). The balance between the accumulation of ROS and the

antioxidant system are partially involved in cell-cycle progression, and interference during cell division may lead to abnormal cell proliferation (35). Our results indicated that NCTD increased the production of ROS in AGS cells. Oxidative stress maybe caused by the overproduction of ROS in cells or tissues and it can be an important mediator of cell structure damages and initiation of cancer development (36).

Numerous evidence has indicated that mitochondria dysfunction reduces Ψ_m , associated with cell apoptosis (37-39). Induction of cell apoptosis through reduction of Ψ_m by some anticancer drugs is mediated through the intrinsic signaling pathway (40, 41). Herein, NCTD significantly reduced the $\Delta\Psi_m$ in AGS cells and these effects were time-dependent. These observations are in agreement with other reports that NCTD induced cancer cell apoptosis is associated with mitochondrial dysfunction (10, 42). Furthermore, NCTD

was shown to induce cell apoptosis through ROS-mediated mitochondrial dysfunction and energy depletion in DU145 prostate cancer cells (10). Therefore, we investigated expression of proteins associated with cell apoptosis which is also related to $\Delta\Psi_m$ in AGS cells after exposure to NCTD *in vitro*. NCTD increased expression of pro-apoptotic proteins such as BAX and BID and increased that of the active form of caspase-9, cytochrome *c*, AIF and Endo G in AGS cells. Based on these findings, we suggest that NCTD induced cell apoptosis of AGS through the mitochondria-dependent pathway by increased ROS production, loss of $\Delta\Psi_m$, increased expression of apoptosis-associated proteins, leading to cell apoptosis.

Numerous studies have shown that the activation of the caspase cascade is associated with cell apoptosis (43, 44). We used flow cytometric assay and found that NCTD increased activity of caspase-9. These results were also confirmed by western blotting. These results suggest that NCTD may induce apoptosis mainly through the activation of caspase-3 and caspase-9. Results from western blotting also showed that NCTD significantly increased the protein expressions of DR4 and DR5 and TNF α in AGS cells, indicating that NCTD induced cell apoptosis of AGS cells may be partially through these signaling pathways.

The present study indicated that NCTD reduced AGS human gastric cancer cell numbers by inducing G₀/G₁ phase arrest and cellular apoptosis *in vitro* (Figure 8). The induction of apoptosis by NCTD was associated with caspase- and mitochondria-dependent pathways and accompanied by the releases of cytochrome *c*, AIF and Endo G.

Conflicts of Interest

The Authors report no conflict of interest in regard to this study.

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