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Research Paper

Norcantharidin activate the mitophagy and triggers apoptotic cell death in non-small cell lung cancer (NSCLC)

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ABSTRACT

This study was designed to examine the efficacy of NCTD on A549 Non-small cell lung cancer (NSCLC) cell death and to elucidate detailed mechanisms of its activity. Norcantharidin (NCTD), a low-toxic analog of the active anticancer compound cantharidin (CTD) in mylabris is isolated from natural blister beetles, can inhibit proliferation and induce apoptosis of multiple types of cancer cells. However, the anticancer activities of NCTD with respect to lung cancer, and its underlying mechanisms, have not been investigated. In this study, NCTD suppressed the proliferation and cloning ability of A549 cells in a dose-dependent manner, apparently by reducing the mitochondrial membrane potential and inducing G2/M phage arrest in A549 cells. NCTD induced apoptosis through increasing the ratio of Bax/Bcl-2 and Bax/Mcl-1 and activating caspase-3/9 dependent mitochondrial pathways. Treatment by NCTD induced significant mitophagy and autophagy, as demonstrated by accumulation of punctate LC3 in the cytoplasm and characteristic clustering of the mitochondria around the nucleus, thereby increasing the protein expression of LC3-II and reducing the protein expression of p62. In addition, decrease in p-AMPK expression accompanied with an increase in p-AKT and mTOR after treatment with NCTD was also observed. In conclusion**,** our results demonstrate that NCTD can reduce the mitochondrial membrane potential, induce mitophagy, and subsequently arouse cellular autophagy and apoptosis; the AMPK-TSC-mTOR signaling pathways are widely involved in these processes. Thus, the traditional Chinese medicine NCTD could be a novel therapeutic strategy for treating Non-small cell lung cancer (NSCLC) cell.

Key words: Norcantharidin, autophagy, apoptosis, non-small cell lung cancer (NSCLC) cell.

INTRODUCTION

Lung cancer is the main cause of all cancer-associated mortalities worldwide (Greenlee et al., 2001). Non-small cell lung cancer (NSCLC) accounts for 75 to 80% of all lung cancer (Siegel et al., 2013). Despite significant advances in diagnosis and treatment, the prognosis of lung cancer is still poor and the mortality rates with an overall 5-year survival of only 15% (Chen et al., 2014). Thus, making it one of the most deadly and difficult cancers in humans to diagnose. To date, chemotherapy has been the most frequently used treatment for lung cancer and other cancers. However, some normal cells are destroyed as well by this method of treatment. Due to their wide range of biological activities and low toxicity in animal models, some natural products have been used as alternative treatments for cancers including lung cancer. Therefore, an urgent need exists for the development of more effective and less toxic anticancer drugs for lung cancer treatment.

Norcantharidin (NCTD) is the demethylated analog of cantharidin isolated from natural blister beetles. The dried body of the Chinese blister beetle (*Mylabris phalerata*

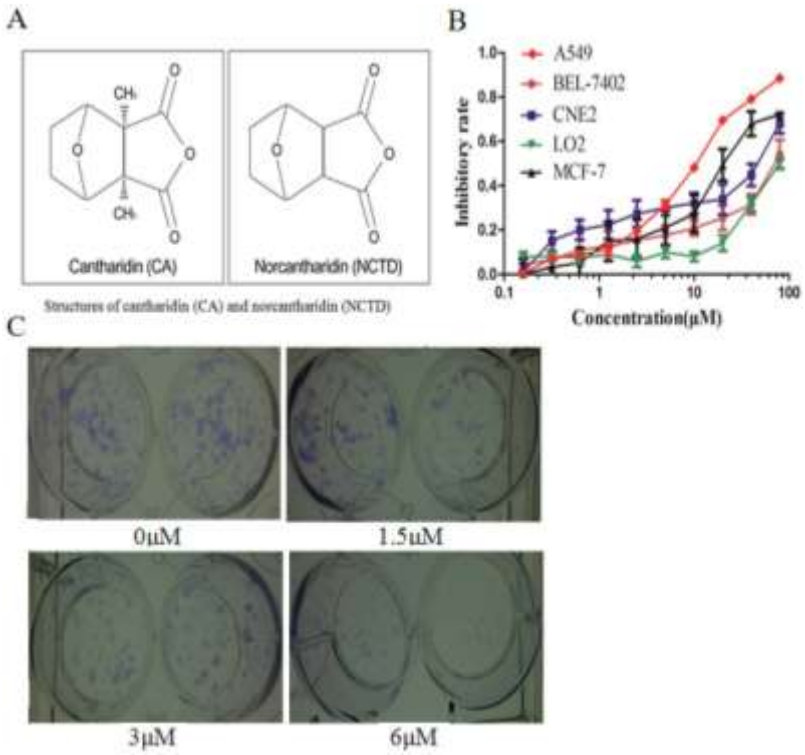

Figure 1: Effects of NCTD on cell viability in A549 cells. (A) NCTD inhibited the proliferation of some cancer cell lines and LO2 cells. They were treated with the indicated concentrations of NCTD for 72 h and cell viability was determined by the MTT assay. (B) Molecular structure of CTD and NCTD. (C) Colony-forming test results. A549 cells were incubated with NCTD (0, 1.5, 3, 6 μM) for 7 days. Data represent one of three experiments yielding similar results.

Pallas), known as Mylabris, is a traditional Chinese medicine (TCM) that has been used for over 2000 years to treat abdominal masses and rabies, and as an abortifacient (Zheng et al., 2016).

Pharmacological studies have revealed that cantharidin (CTD), an active constituent of Mylabris, has anti-tumor properties and causes leukocytosis. However, its applications are limited by its gastrointestinal and urinary tracts side effects (Ren et al., 2016). Norcantharidin (NCTD), a demethylated derivative of CTD (Figure 1B), has been synthesized as a replacement for CTD, to reduce toxic side effects while still retaining the efficacy of CTD. Currently, NCTD is widely used in China as an anti-tumor drug for inhibiting proliferation and metastases of several kinds of carcinomas, including primary heap toma, colorectal cancer, breast cancer, prostate cancer, and lung cancer etc (Mo et al., 2018). Unlike conventional chemotherapeutics, NCTD is preferentially toxic to cancer cells rather than normal cells, making this compound a promising cancer treatment agent. The effect of NCTD in inducing the apoptosis of multiple categories of tumor cells has been frequently cited. However, the relationship between Non-small cell lung cancer (NSCLC) and NCTD remains not thoroughly elucidated (Han et al., 2016).

A direct relationship was suggested to have being in existence between NCTD and autophagy, however, the molecular mechanism is poorly understood. Accumulating evidence has demonstrated the widespread prospect of treating malignant tumors through autophagic regulation. The mitophagic process is initiated to preserve cell life but if cell injury is prolonged, mitophagy can also cause cell death (Tian et al., 2016). Because of this dual function as a tumor suppressor as well as, a promoter of tumor cell survival, establishing the relationship between mitophagy and cancer clearly and completely is not an easy task. The depolarization of mitochondria is a vital early event in mitophagy (Gelmetti et al., 2017). Accumulating evidence has demonstrated the widespread prospect of treating malignant tumors through autophagic regulation. Persistent mitophagy eventually leads to cell autophagy. The effect of mitophagy has been widely studied, and is considered to be an early manifestation of cell autophagy. Mitochondrial damage compromises mitochondrial functions, thereby inducing mitophagy (Korolchuk et al., 2017). It was observed from this study that NCTD induced a dose-dependent decrease of $\Delta\Psi m$ in A549 cells to induce mitophagy and autophagy in A549 cells. Excessive autophagy is likely to accelerate apoptosis promoting cell death (Chen et al., 2018).

To gain further insights into the mechanism of NCTD

induced A549 cell death, we examined the effects of NCTD treatment on cell signaling pathways involved in autophagy and apoptosis and found that NCTD activated AKT induced both autophagy and mitophagy. This is the first study to demonstrate cytotoxic activity and underlying mechanisms of NCTD with respect to A549 Non-small cell lung cancer (NSCLC) cells *in vitro*.

Cell cultures and reagents

Non-small cell lung cancer cell line A549 (Shanghai Institute of Cell Biology, China) was maintained in RPMI medium 1640 (Life Technologies Corporation, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (Gibco Corporation, Carlsbad, CA,USA), 1 mM glutamine, 1% penicillin/streptomycin, 1.5 g/L sodium bicarbonate, and 1 mM sodium pyruvate at 37°C and 5% CO_2. The primary antibodies used for immunoblots included anti-Bcl-2 anti-Bax, anti-PARP-1, anti-Mcl-1, anti-Bcl-xL, anti-Bcl-2, anti-Bax, anti-caspase-3, anti-caspase-8, anti-caspase-9, anti-PARP, anti-AMPK, anti- p-AMPK, anti-Akt, anti-p-Akt, anti-c-Jun, p-c-Jun, anti-JNK, anti-GSK, anti-P-GSK, anti-FKHRL1, anti-P-FKHRL1, anti-ULK1, anti-p-JNK, anti- mTOR, anti-Beclin-1, anti-LC3, anti-p62 and anti-GAPDH; anti-DAPI, and anti-Tom20 were purchased from Cell Signaling Technology Inc., while anti-cyclin A, anti-cyclin B2, anti-cyclin D1, anti-cyclin D3, anti-cyclin E2, anti-p21, anti-Myt1, anti-Cdc2, and anti-p-Cdc2 were purchased from Sigma-Aldrich. Analytical grade NCTD was purchased from Sigma-Aldrich (St. Louis, MO, USA). A stock solution of NCTD (60 mM) in Dulbecco's modified eagle medium (DMEM) (Invitrogen, Victoria, Australia), was prepared and stored at -20°C. Cell growth inhibition was determined using the 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) assay, as previously described (Mclafferty et al., 2016).

Cell proliferation assay

Cell proliferation was evaluated by the MTT assay. Cells were plated in 96-well plates at a density of 5×10^3 cells/ml. After 24 h, NCTD was added at final concentrations of 0, 0.16075, 0.3125, 0.625, 1.25, 2.5, 5, 10, 20, 40, and 80 μM, respectively. Cells without NCTD treatment comprised the control group. Cells were incubated with various concentrations of drugs for 72 h, followed by the addition of MTT solution (10.0 μl/ well) and incubation for a further 4 to 6 h at 37°C in a humidified 5% CO_2 atmosphere. At the end of the incubation period, the purple formazan crystals were dissolved in 200 μl dimethyl sulfoxide (DMSO). After the crystals had dissolved, the plates were analyzed on an automated microplate spectrophotometer (Thermo Molecular De-vices Co., Union City, USA) at 570 nm. The inhibition rate of cell proliferation was calculated for each well as ($A570_{\text{control cells}} - A570_{\text{treated cells}}$)/$A570_{\text{control cells}} \times 100\%$. The IC_{50} values were determined by the logit method. Experiments were performed in triplicate.

Clone formation assay

Clone formation assay was used to evaluate the effects of NCTD on the proliferation of A549 cells. The A549 cells were cultured in 12-well microplates (300 cells/ well) in 2 ml of 100% 1640 for 24 h. Thereafter, cells were treated with indicated concentrations of NCTD in microplates for 7 days. Cells were stained with crystal violet (Sigma, St. Louis, Mo, USA) for 20 min. Images of the colonies were obtained using a digital camera (Canon, EOS350D, Tokyo, Japan).

Cell apoptosis and death analysis

Flow cytometry (Beckman Coulter, Fullerton, California, USA) was used to identify apoptotic cells by DNA fragmentation analysis. To determine the apoptotic rate, A549 cells were placed in 6-well plates and treated with 0, 5, 10, 20, and 40 μM of NCTD at a density of 2×10^5 cells/well for 24 h, respectively and then collected. Annexin V- FITC/PI (Becton Dickinson, USA) staining was performed, following the manufacturer's protocol. Phosphatidyl serine translocation to the cell surface is an indicator of early apoptotic cells. Therefore, annexin V-positive PI-negative cells were identified as apoptotic. The apoptotic rate was determined using CellQuest software (FCM, Becton Dickinson, USA).

Cell cycle analysis

The cell cycle distribution was determined by DNA staining with propidium iodide (PI) (Becton Dickinson, USA). Briefly, A549 cells were incubated with 0, 5, 10, 20, and 40 μM of NCTD for 24 h, respectively. Approximately, 2×10^5 cells were collected and fixed in 70% ice-cold ethanol overnight. The fixed cells were incubated with 20 units/ml RNase I and 50 μg/ml propidium iodide for 30 min. Thereafter, the cellular DNA content was determined by flow cytometry and the results analyzed using CellQuest software (FCM, Becton Dickinson, USA).

Measurements of mitochondrial membrane potential (ΔΨm)

The mitochondrial membrane potential (ΔΨm), which detects mitochondrial depolarization by a change in the fluorescence intensity from red to green was measured using JC-1 dye (Beyotime Co, Hangzhou, China). The A549

cells were placed in 6-well plates and treated with 0, 5, 10, 20, and 40 μM NCTD at a density of 2 × 10^5 cells/well for 24 h, respectively. Cells were re-suspended and then detected by flow cytometry, or harvested and smeared on slides. Images were obtained under a fluorescent microscope (DFC480; Leica Microsystems, Wetzlar, Germany).

Western blot assays

A549 cells were treated with 0, 5, 10, 20 and 40 μM of NCTD for 24 h, respectively. Whole cell lysates were prepared by adding 5×SDS sample buffer. Equal amounts of protein were electrophoresed on 10% SDS-PAGE gels and transferred to PVDF membranes. The membranes were then blocked for 60 min at room temperature with 5% non-fat dry milk/TBST (TBS-Tween 20) and reacted with appropriate antibodies for Bcl-2, Bax, PARP, Mcl-1, Bcl-xL, Bcl-2, Bax, caspase-3, caspase-8, caspase-9, AMPK, p-AMPK, Akt, p-Akt, c-Jun, p-c-Jun, JNK, GSK, p-GSK, FKHRL1, p-FKHRL1, ULK1, p-JNK, mTOR, Beclin-1, LC3, p62, cyclinA, cyclinB2, cyclinD1, cyclinD3, cyclinE2, p21, Myt1, Cdc2, p-Cdc2 and GAPDH (1:1000 dilution in blocking buffer) at 4°C overnight, respectively. Following incubation with the primary antibody, membranes were washed in TBST and incubated with horseradish peroxidase-conjugated secondary antibodies for 1 h at room temperature. Proteins were visualized by incubation with SuperSignal west pico reagents (NCI5079, Thermo), followed by exposure to radiograph film.

Immunocytochemistry

Immunofluorescence staining was conducted using a procedure similar to that described previously (Lalaoui et al., 2015). The A549 cells were plated on sterile coverslips and treated with 0μM NCTD (control group) and 5 μM NCTD for 24 h, and fixed with 4% paraformaldehyde (PFA) for 10 min at 37°C. After fixation, a permeabilization step was conducted with 0.25% Triton-X 100 for 10 min at 4°C, and the cells were subsequently incubated in blocking solution containing 4% bovine serum albumin (BSA) for 1 h at 37°C. Cells were then incubated with LC3 antibody (1:200 dilution in blocking buffer) or Tom20 antibody (1:200 dilution in blocking buffer) for 24h at 4°C followed by FITC-conjugated secondary antibody for 2 h at room temperature. The nucleus was stained with DAPI (1 mg/ml) for 5 min at room temperature. Fluorescence images were then captured by a confocal laser scanning microscope (LSM 700) (Carl Zeiss, Oberkochen, Germany).

Statistical analysis

All assays were performed in triplicate. Data are expressed as the mean ± SD. Statistical analyses were performed using an analysis of variance with SPSS 13.0 software.

RESULTS

Growth inhibition effects of NCTD on some cancer cell lines and LO2 cells

To examine whether NCTD has a cytotoxic effect on human cancer cells, we assessed the viability of human cancer cell lines (Non-small cell lung cancer cell line A549, neuroblastoma cell line SKNSH, Breast cancer cell line MCF-7, hepatocellular carcinoma cell line BEL-7402, nasopharyngeal carcinoma cell line CNE-2) and human hepatic cell line LO2 cells. Cells were exposed to different concentrations of NCTD for 72 h, and as shown in Figure 1, NCTD exhibited a broad spectrum of growth inhibition and cytotoxicity against these cancer cell lines and it showed a moderate cytotoxic activity on normal LO2 cells. These results indicate that NCTD strongly inhibited the growth of these tumor cells in a dose- and time-dependent manner (Figure 1A), and the inhibitory effect of NCTD on A549 cells show that NCTD significantly inhibited the viability of A549 cells in a dose-dependent manner, after cells were exposed for 72 h to varying concentrations of NCTD (Figure 1A); the IC_{50} value was 1 μM. In addition, colony formation assay showed that NCTD strongly inhibited the proliferation of A549 cells (Figure 1C).

NCTD arrested A549 cells at the G2/M stage and affected the expression of cell cycle proteins

To delineate the mechanisms responsible for the inhibition of cell proliferation by NCTD, we examined the cell-cycle distribution using flow cytometry. Results showed that the cycle progression was significantly inhibited at the G2/M phase, as presented in Figure 2A. To gain further information, cell-cycle regulatory protein expressions were investigated by Western blot. The results showed that NCTD treatment led to a significant dose-dependent decrease in the levels of Myt1, Cdc2, and also the phosphorylation of Cdc2, but p21 expression was elevated (Figure 2B). Moreover, NCTD treatment caused a pronounced decrease in the expression of cyclin D1, cyclin D3, cyclin E2, and cyclin B1, while cyclin A expression increased inversely (Figure 2C).

NCTD induced apoptosis dose-dependently and reduced the membrane potential of mitochondria in A549 cells

To investigate the mechanism of NCTD-inhibition of cell

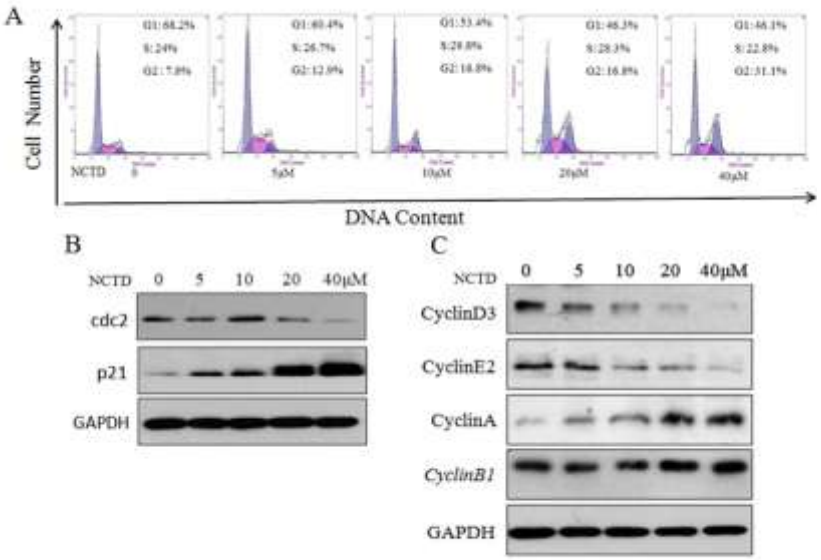

Figure 2: NCTD induced cell cycle arrest of A549 cells. (A) A549 cells were exposed to various concentrations of NCTD (0, 5, 10, 20, 40 μM) for 24 h followed by analysis of cell cycle by flow cytometry. (B) Western blot analysis of cell cycle-associated protein extracts obtained from A549 cells treated with different concentrations of NCTD. (C) Cyclin family members were tested after NCTD treatment as described.

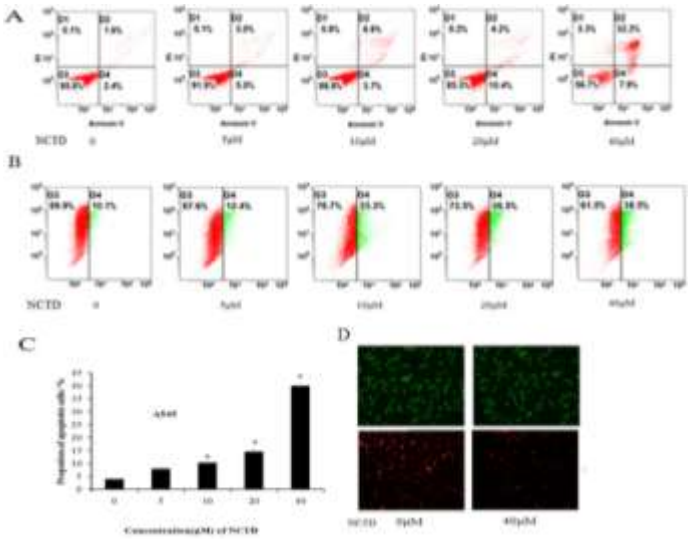

Figure 3: Induction of apoptosis and reduction of mitochondrial membrane potential in A549 cells by NCTD. (A) NCTD-induced apoptosis determined by annexin V-FITC and PI staining. (B) The change in mitochondrial membrane potential after 0,5,10,20,40 μM of NCTD explosion was evaluated by flow cytometry method. (C) Fluorescence microscopy was used to determine the mitochondrial membrane potential after different concentration (0, 40 μM) of NCTD treatment. Data shown are representative of three different experiments.

viability, the effects of NCTD on cell apoptosis using flow cytometry was analyzed. Annexin V(+)/PI(−) and annexin V(+)/PI(+) represent cells in early apoptosis and late apoptosis/necrosis, respectively. As shown in Figure 3A and B, the total apoptosis proportion in A549 cells increased as the NCTD concentration increased from 4.0 to 40.1% after treatment, suggesting that NCTD induced apoptosis in A549 cells dose-dependently, especially late apoptosis.

Loss of mitochondrial membrane potential ($\Delta\Psi m$) is a crucial step in the process of cell death. In apoptosis cells, $\Delta\Psi m$ and mitochondrial integrity are irreversibly compromised. Consequently, we chose the cationic dye JC-1 to examine the role of mitochondria in cell death using the flow cytometry method. We used a computer-based program to calculate the ratio of green to red fluorescence after 24 h of treatment at varying concentrations of NCTD. Figure 3C shows that NCTD induced a dose-dependent decrease of $\Delta\Psi m$ in A549 cells. The JC-1 aggregates showed a spectral shift in emitted light from 530 nm (that is, green-colored emissions of the monomeric JC-1 form) to 590 nm (that is, green-orange-colored emissions of the J-aggregate) upon excitation at 490 nm. Under fluorescence microscopy, the cells treated for 24 h with 20 and 40 μM NCTD both showed high green and low orange fluorescence, as compared with the control group, which showed high green and high orange fluorescence (Figure 3D).

NCTD changed expression levels of Bcl-2 family proteins and activated caspase-3/9/PARP in A549 cells

To investigate the role of Bcl-2 family in NCTD-induced apoptosis, the protein expression levels by Western

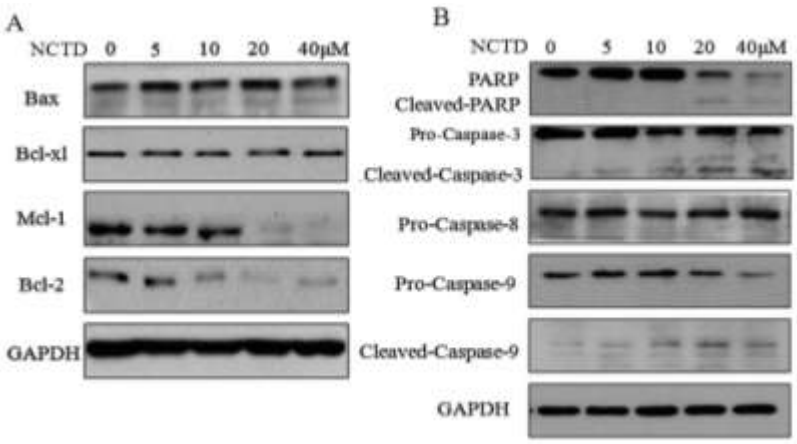

Figure 4: NCTD changed expression levels of Bcl-2 family proteins and activated caspase-3/9/PARP in A549 cells. (A) NCTD inhibited Bcl-2 family members. A549 cells were treated as previously described, and the effect of NCTD on Bcl-2 family members expression level was determined by western blot. (B) NCTD activated caspase-3 and -9 and RAPR. A549 cells were treated with 0, 5, 10, 20, 40 μM NCTD for 24 h. Data represent one of three experiments yielding similar results.

blotting was determined. As show in Figure 4A, treatment with NCTD for 24 h markedly down-regulated the expression levels of the anti-apoptotic proteins Bcl-2 and Mcl-1. However, NCTD treatment of the A549 cells did not obviously alter the expressions of two other pro-apoptotic proteins, Bax and Bcl-xl, as observed in Figure 4A.

Caspases was also activated after NCTD treatment. Figure 4B shows that NCTD activated caspase-3 and caspase-9 in a dose-dependent manner, while expression levels of pro-caspase-8 were not significantly changed. Furthermore, PARP apoptotic fragments were generated after treatments with 20 and 40 μM NCTD, respectively.

NCTD-induced autophagy and mitophagy

To validate NCTD-induced autophagy of A549 cells, the expression levels of LC3, and p62 were measured by Western blotting after treatment of A549 cells with NCTD. As shown in Figure 5A, the treatment of A549 cells with NCTD accelerates the differentiation of LC3-I into LC3-II and reduced the protein expression of p62. To make further validation, the formation of autophagosome was observed under a confocal microscope. LC3 as an autophagosome membrane marker, was demonstrated by accumulation of punctate LC3 in the cytoplasm of A549 cells (Figure 5C) hinting that NCTD induced the autophagy of A549 cells. NCTD activated loss of mitochondrial membrane potential ($\Delta\Psi m$), is a crucial step in the process of mitophagy. This study investigated whether mitophagy occurs after NCTD treatment, to further explore the mechanism underlying NCTD-induced autophagy of A549 cells. The TOM20 protein is a mitochondrial outer membrane protein, and therefore reductions in its expression levels can reflect mitophagy (Zhuang et al., 2008). The expression levels of TOM20 were measured by Western blotting after treatment of A549 cells with NCTD. As shown in Figure 5B, the treatment of A549 cells with NCTD reduced the protein expression of TOM20. Figure 5D shows the characteristic clustering of the mitochondria around the nucleus indicated that NCTD induced mitophagy in A549 cells.

Effect of NCTD on expression of intracellular signaling proteins

The upstream of mTOR signaling was mainly through PI3K / AKT / mTOR pathway and non-dependent PI3K / AKT pathway, in order to achieve a variety of physiological functions of cell growth, and cell cycle regulation. The upstream of mTOR signaling pathway was in order to regulate a variety of physiological functions which including cell growth, cell cycle mainly through PI3K / AKT / mTOR pathway and non-dependent PI3K/AKT pathway; AKT is up-stream regulated kinase of AMPK, belonging to the serine / threonine protein kinase sub-family. AKT inhibits AMPK and through AMPK-TSC-MTOR way to activate mTOR (Lalaoui et al., 2015; Zhao et al., 2017). Two conditions are required to activate Akt: (1) AKT need translocation to the plasma membrane (2) AKTDE Thr308, ser473 phosphorylation (Zhuang et al., 2008). The main functions of activated Akt: (1) akt through the phosphorylation of transcription factor FOXO (including FKHR, FKHRL1, and AFX three members), Pro-caspase-9 play an anti-apoptotic effect (2) Akt phosphorylate the glycogen synthase (GSK-3), p21 WAF1, while p27 Kip1

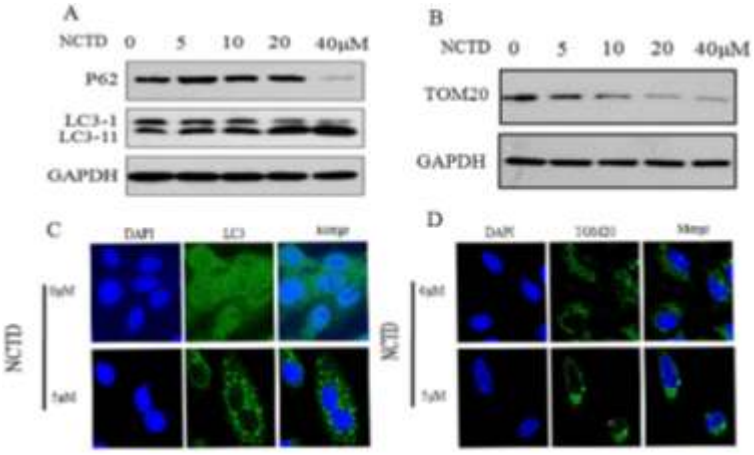

Figure 5: NCTD induced autophagy and mitophagy in A549 cells. (A) The expression of LC3 and p62 were performed by western blotting. A549 cells were treated with NCTD (0, 5, 10, 20, 40 μM) for 24 h. (B) Punctuates of LC3 proteins in NCTD-induced A549 cells. Cells were incubated with NCTD (0, 5μM) for 24 h and then stained with the anti-LC3 antibody. (C) NCTD induced mitophagy in A549 cells. Different concentration of NCTD (0, 5μM) treated A549 cells for 24 h, and stained with the anti-TOM20. Cells were examined by fluorescence confocal microscopy. Green: FITC-labeled LC3 or TOM20; Blue: DAPI-labeled nucleus. Magnification: ×200.

accelerated cell cycle progression (Yin et al., 2016). Some anti-tumor medications reportedly activate Akt and inhibit AKT/mTOR signaling pathways and induce autophagy and apoptosis of cancer cells (Garcíaprat et al., 2016; Avalos et al., 2014). Hence, expression of mTOR and phosphorylated levels of Akt and AMPK in A549 cells treated with NCTD were analyzed by Western blotting. Figure 6A shows that expression of mTOR did not significantly change after treatment with a low concentration of NCTD, whereas levels declined considerably after treatment with 40 μM NCTD. The expression of p-AKT was down-regulated whereas those of p-AMPK were up-regulated with increasing concentrations of NCTD. Moreover, previous studies demonstrated that NCTD-induced apoptosis of tumor cells is accompanied by activation of the JNK signaling pathway (Rehman et al., 2014; De et al., 2016). Western blotting revealed that p-JNK, p-GSK, p-FKHRL1 and p-c-Jun expression levels of were elevated in a dose-dependent manner (Figure 6B), suggesting that NCTD participates in the apoptosis of A549 cells.

DISCUSSION

Apoptosis, also known as programmed cell death, is a genetically regulated, self-destructive cellular death process that is important in development, tissue remodeling, immune regulation, and several diseases (Redza-Dutordoir and Averill-Bates, 2016). Cysteine-dependent aspartate-specific proteases (caspases) have been demonstrated to be crucial mediators in apoptotic pathway. Caspases can be divided into two groups: initiator caspases (such as caspase-8 and caspase-9) whose main function is to activate downstream caspases, and executor caspases (such as caspase-3), which mediate apoptosis by proteolysis of specific substrates including inhibitor of caspase-activated DNase (ICAD) and antiapoptotic protein, Bcl-2 (Galluzzi et al., 2016). Many Bcl-2 family proteins reside in the mitochondrial outer membrane. Bcl-2 and Bcl-xL can be cleaved by caspase-3 and cleavage of these proteins appears to inactivate their survival function. In response to the death stimuli, the mitochrondrial membranes are permeabilized, resulting in the release of cytochrome *c*. In the cytosol, cytochrome *c* activates apoptosis by binding and activating apoptotic protease activating factor-1 (Apaf-1)-caspase-9 complex, which forms an apoptosome acting as a processing/activation center for the downstream caspase-3 (Carthy et al., 2016).

There are numerous citations regarding the effect of NCTD in inducing the apoptosis of multiple categories of tumor cells. Previous studies demonstrated that NCTD induces an inhibitory effect upon the growth and proliferation of multiple tumor cells in a dose-dependent manner. However, until now, no study has examined the treatment of Non-small cell lung cancer (NSCLC) with NCTD. In this study, A549 Non-small cell lung cancer (NSCLC) cells were treated with varying concentrations of NCTD to investigate the molecular mechanisms underlying the inhibitory effect of NCTD on the growth of tumor cells. An MTT assay revealed that NCTD can significantly suppress the growth of A549 cells in a dose-dependent manner. A clone formation assay revealed that the number of cell

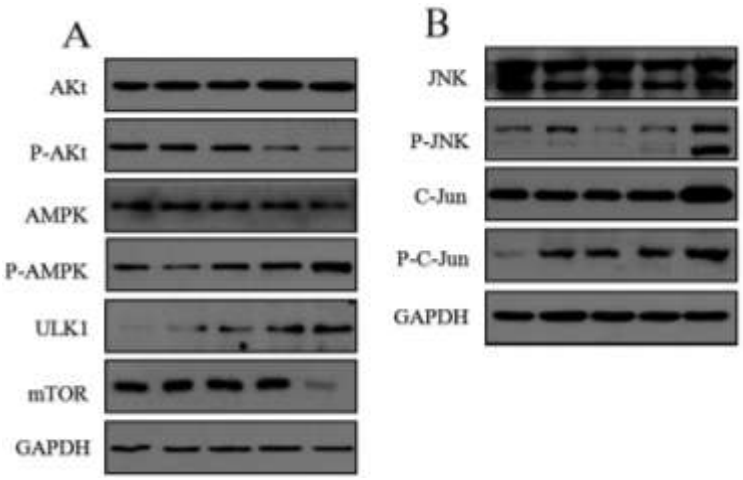

Figure 6: Effects of NCTD on intracellular signaling protein expression levels in A549 cells. (A) NCTD activates the autophagy signaling pathway in A549 cell. A549 cells were treated with 0, 5, 10, 20 and 40 μM NCTD for 24 h. The effects of NCTD on p-AMPK , p-Akt ,ULK1 and mTOR expression levels were evaluated by western blotting. (B) NCTD activates the apoptosis signaling pathway in A549 cells. After treatment, A549 cells were subjected to western blotting for JNK, p-JNK, c-Jun,p-c-Jun,GSK,p-GSK,FKHRL1, p-FKHRL1, Both experiments were individually performed three times and present representative data.

clones formed in the presence of NCTD decreased with increasing NCTD concentration, suggesting that NCTD exerts an inhibitory effect on cell growth (Figure 1).

The inhibitory effect of NCTD was ascribed to the promotion of cell apoptosis by NCTD. Apoptosis has been widely studied in relation to the treatment of malignant tumors. In accordance with these studies, our flow cytometry results showed that NCTD can effectively induce apoptosis of A549 cells in a dose-dependent manner (Figure 3A and B). Bcl-2 and caspase family members play crucial roles in cell apoptosis. Levels of Bcl-2, Bcl-xl, and Mcl-1 on the mitochondrial membrane decreased, leading to an increase in Bax/Bcl-2, Bax/Bcl-xL, and Bax/Mcl-1 (Figure 3A). Thus, NCTD induced a dose-dependent decrease of $\Delta\Psi m$ in A549 cells (Figure 2C and D). The permeability of the outer mitochondrial membrane is enhanced, while the mitochondrial membrane potential is decreased, releasing apoptosis-promoting substances, such as, from the mitochondria to initiate the activation of caspase-9, which eventually activated downstream effector caspase-3 leading to cell death (Mclafferty et al., 2016).

In this study, the expressions of Bcl-2 and Mcl-1 decreased with increasing concentrations of NCTD. However, Bax and Bcl-xl were insensitive to NCTD, resulting in an increase in Bax/Bcl-2 and Bax/Mcl-1 in the presence of NCTD, and in so doing cleaved active caspase-9 and caspase-3 were demonstrated (Figure 4A). The activated caspase-3 cleaves PARP and causes morphological and biological apoptotic changes, which eventually inhibits proliferation and accelerate apoptosis of A549 cells. As no obvious changes were observed in caspase-8 levels, we speculate that A549 cell apoptosis induced by NCTD does not involve exogenous apoptosis pathways (Figure 4B). As is well known, almost all types of malignant tumors are characterized by abnormal cell proliferation caused by cell cycle disorders (Lalaoui et al., 2015). Several lines of evidence from this study have suggested that NCTD-induced apoptosis in human tumor cells might be associated with arrest of the cell cycle at the G2/M phase through changes in the expression of cell cycle-related genes, including cyclins, cyclin-dependent kinases (CDKs), and cyclin-dependent kinase inhibitors (CDKIs; such as p21) (Zhao et al., 2017).

In the present study, Western blot analysis revealed that expressions of cyclin D1, cyclin D3, cyclin E2, and cyclin B1 were down-regulated, whereas the expression of cyclin A was up-regulated (Figure 2C), indicating that the number of cells at the G2/M stage gradually increased with increasing NCTD concentrations, which is consistent with the findings of flow cytometry (Figure 2A), suggesting that NCTD is able to inhibit the proliferation of A549 cells probably by interfering with cellular mitosis. Among Cip/Kip family members, p21 and p27 can interact with CDK and cyclin compounds to arrest cells at the G1 or G2 stages. The transcription factor, Myt1 can result in phosphorylation of Cdc2 at Tyr15 and Thr14, thereby arresting cells at the G2 stage. It was reported that AMPK can suppress the cell cycle and affect cell proliferation by up-regulating the expression of p21 protein (Zhuang et al., 2008). Our results indicate that NCTD treatment leads to a significantly dose-dependent decrease in levels of Myt1, Cdc2, and p-Cdc2, but that expression of p21 is elevated (Figure 2B), hinting that NCTD activates AMPK, inhibits AKT, up-regulates p21 expression,

and suppresses the activity of cyclin B1 and Cdc2, thus, arresting cells at the G2/M stage. At the same time, increased expression of Myt-1 causes Tyr15 phosphorylation of Cdc2, suppressed Cdc2 activity, and decreased levels of cyclin B1 and Cdc2, thereby also arresting A549 cells at the G2/M stage.

Autophagy is primarily a process for cell protection, playing a pivotal role in cell survival, differentiation, development, and homeostasis. Autophagy, also known as type II programmed cell death, is an independent cell death process different from apoptosis (Yin et al., 2016). However, unlimited autophagy tends to gradually consume intracellular components and arouse cell death (Garcíaprat et al., 2016). Certain anti-tumor drugs have been proven to induce autophagy (Avalos et al., 2014) and the anti-tumor effects of autophagy on tumor cells have captivated increasing attention among oncologists. Excessive autophagy is likely to accelerate apoptosis promoting cell death (Rehman et al., 2014). Subsequently, we demonstrated that treatment of A549 cells with NCTD can be induced to increase the protein expression of LC3-II and reduce the protein expression of p62 (Figure 5A), and the accumulation of punctate LC3 in the cytoplasm of A549 cells (Figure 5C), hinting that NCTD induced the autophagy of A549 cells.

Mitophagy, a selective form of macroautophagy leading to cell autophagy, refers to the scavenging of injured mitochondria by an autophagosome, and then fusing of the autophagosome with a lysosome and its subsequent degradation, which completes the degradation of the injured mitochondria and thus maintains the stability of the intracellular environment (De et al., 2016). Mitophagy, which is a mitochondrial recycling process that primarily promotes cell survival, can also cause cell death in the presence of prolonged injury. Accumulating evidence has demonstrated the widespread prospect of treating malignant tumors through autophagic regulation. Persistent mitophagy eventually leads to cell autophagy. The depolarization of mitochondria is a vital early event in mitophagy (De et al., 2016). The effect of mitophagy has been widely studied, and is considered to be an early manifestation of cell autophagy. Mitochondrial damage compromises mitochondrial functions, thereby inducing mitophagy (Lemus et al., 2016). It was observed that NCTD induced a dose-dependent decrease of ΔΨm in A549 cells (Figure 3C, and D). The TOM20 protein is a mitochondrial outer membrane protein, and therefore reductions in its expression levels can reflect mitophagy (Powell et al., 2016). The expression levels of TOM20 were measured by Western blotting after treatment of A549 cells with NCTD. Figure 4B shows that the treatment of A549 cells with NCTD reduced the protein expression of TOM20 and characteristic clustering of the mitochondria around the nucleus (Figure 5D) indicating that NCTD induced mitophagy in A549 cells.

mTOR as a key adjustment factor in autophagy, after its activation inhibits autophagy (Dilly et al., 2013). The upstream of mTOR signaling pathway, in order to regulate a variety of physiological functions including cell growth, cell cycle is mainly through PI3K / AKT / mTOR pathway and non-dependent PI3K / AKT path-way; AKT is upstream regulated kinase of AMPK, belonging to the serine / threonine protein kinase sub-family. AKT inhibits AMPK and through AMPK-TSC-MTOR way to activate mTOR (Lalaoui et al., 2015; Zhao et al., 2017). The two conditions required to activate Akt are: (1) AKT need translocation to the plasma membrane; (2) AKTDE Thr308, and ser473 phosphorylation (Zhuang et al., 2008). The main function of activated Akt: (1) akt through the phosphorylation of transcription factor FOXO (inclueing FKHR, FKHRL1, and AFX three members), Pro-caspase-9 play an anti-apoptotic effect; (2) Akt though phosphorylate the glycogen synthase (GSK-3), p21 WAF1 and p27 Kip1 accelerated cell cycle progression (Dilly et al., 2013).

Previous studies demonstrated that NCTD is capable of activating AMPK in mammalian animal cells. AMPK, a protein kinase that senses energy status, is vital to the regulation of metabolism, autophagy, and apoptosis (Lin et al., 2017). As a signaling pathway molecule, AMPK is able to induce autophagy directly through the ULK1 protein or indirectly by inhibiting the mTOR signaling pathway (Yuan et al., 2018). In this study, NCTD activated Akt through up-regulate p-AMPK and suppressed the expression of mTOR, indicating that NCTD is associated with autophagy (Figure 5A).

Recent studies demonstrated that the incidence of multiple diseases could involve activation of JNK/c-Jun, reduced expressions of Bcl-2 and Mcl-1, and accelerated release of mitochondrial cytochrome C into cytoplasm, thereby leading to caspase activation and cell apoptosis (Ju et al., 2013). Consequently, we confirmed that NCTD participates in the autophagy and apoptosis of A549 cells by activating the AMPK-TSC-MTOR signaling pathway (Figure 5A, and B). Previous research demonstrated that the AKT/mTOR signaling pathway is critical to cell proliferation, and that activation of this pathway is correlated with the incidence and progression of varying malignant tumors (Beck et al., 2014). Some anti-tumor drugs can induce early autophagy and late apoptosis of tumor cells through the Akt/mTOR signaling pathway (Zhong et al., 2016). Activated Akt can activate Bcl-2 or Bcl-xl, suppress the release of cytochrome C, and inhibit caspase proteinase, thereby suppressing the incidence of apoptosis (Figure 4), corresponding to the expression level of ULK1 and mTOR, indicating that NCTD mediates autophagy of A549 cells through the Akt/mTOR signaling pathway (Figure 5).

Taken together, Figure 7 shows that NCTD treatment first activates AKT, and then the downstream signaling pathways, which causes the incidence of cell autophagy and cell apoptosis through mechanisms that involve activation of the Akt signaling pathway and inhibition of mTOR

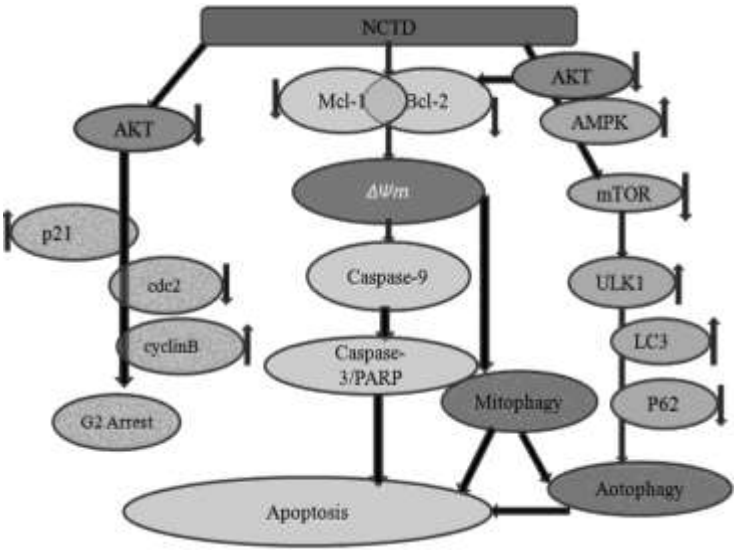

Figure 7: Schematic diagrams showing the mechanisms underlying NCTD induced A549 cells death.

expression. The suppression of Akt phosphorylation accelerates the incidence of autophagy by down-regulating the expression of mTOR, as activation of this pathway not only up-regulates the expressions of P-AMPK, ULK1 and LC3-II, down-regulates the expression of mTOR and p62, and participates in the incidence of autophagy. On the other hand, Akt may suppress the expression of apoptosis-related protein Bcl-2 and Mcl-1 through mitochondrial pathways, resulting in the activation of caspase-3 and caspase-9, leading to cleavage of PARP and eventually causing apoptosis of A549 cells. Negative regulation of Bcl-2, and Mcl-1 reduces the mitochondrial membrane potential and initiates a program of mitophagy. The activation of mitophagy equally participates in autophagy and apoptosis of A549 cells. In addition, the activation of the JNK/c-Jun signaling pathway is also involved in NCTD-induced apoptosis of A549 cells. Consequently, NCTD is able to induce autophagy and apoptosis of Non-small cell lung cancer (NSCLC) A549 cells through multiple signaling pathways. We conclude that NCTD may provide avenue for the development of novel therapeutics.

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