# A mitophagy-mediated autophagy pathway that triggers apoptotic cell death in non-small cell lung cancer (NSCLC) for anticancer chemotherapy by Norcantharidin

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**Abstract**

Norcantharidin (NCTD) is a traditional Chinese medicine (TCM) that has been used for over 2000 years to treat many diseases. It can inhibit proliferation and induce apoptosis in multiple types of cancer cells. However, the anticancer activities of NCTD in non-small cell lung cancer (NSCLC) cells, and its underlying mechanisms, have not been investigated. In this study, NCTD suppressed the growth and proliferation of A549 non-small cell lung cancer cells in a dose-dependent manner, apparently by reducing the mitochondrial membrane potential and inducing G2/M phase arrest. NCTD induced apoptosis by increasing the ratio of Bax/Bcl-2 and Bax/Mcl-1 and activating caspase-3/9-dependent mitochondrial pathways. Treatment with NCTD induced significant mitophagy and autophagy, as the demonstrated by accumulation of punctate LC3 in the cytoplasm and the characteristic clustering of the mitochondria around the nucleus, increasing the conversion of LC3-I to LC3-II and reducing the protein expression of p62. In addition, we also observed an increase in p-AMPK, p-JNK and p-c-jun and decrease in p-AKT and mTOR. In conclusion, our results demonstrate that NCTD can reduce the mitochondrial membrane potential and subsequently increase cellular autophagy and apoptosis; the AMPK/mTOR/ULK1, JNK and Akt/mTOR signaling pathways were involved in these processes. Thus, the traditional Chinese medicine NCTD could be a novel therapeutic for treating NSCLC cells.

## Introduction

Lung cancer is the leading cause of cancer-associated mortalities worldwide[1]. Non-small cell lung cancer (NSCLC) accounts for 70-80% in all lung cancer cases [2]. Despite significant advances in diagnosis and treatment, the prognosis of lung cancer is still poor, with an overall 5-year survival rate of only 15%[3], making it one of the most deadly cancers in humans. In nowdays, chemotherapy has become the most commonly used treatment for lung cancer. However, chemotherapy has numerous adverse effects, including Multiplesystem multiple organ damage including Circulatory system, blood system, digestivesystem. Therefore, there is an urgent need for High effective and low toxic drugs for lung cancer treatment.

Norcantharidin (NCTD), a less-toxicity anabolic hormone analogue (CTD),is found in mylabris. Pharmacological studies have showed that CTD's application is limited by gastrointestinal and urinary tract side effects [4]. A demethylated derivative of CTD, Norcantharidin (NCTD), was synthesized (Figure 1A) to replace CTD to reduce toxic side effects while still retaining the efficacy of CTD. Currently, NCTD has been widely used in China as an anti-tumor treatment. NCTD is more toxic to cancer cells than normal cells, which is different from traditional chemotherapy [5], Making this compound a potential for anticancer therapeutics.

The NCTD has been showed to be related with apoptosis in sorts of cancers .Yet, the relationship between non-small cell lung cancer (NSCLC) and NCTD has not been thoroughly elucidated, although NCTD is involved in autophagy, the molecular mechanism is poorly understood. Previous studies have shown that autophagy plays an important role in the development of malignant tumors. Mitochondrial depolarization is a crucial early event in mitochondria[6].The mitophagic process is initiated to maintain cell line survival, but if cell is excessively damaged, mitophagy can also cause cell death[7]. In our study, we found that NCTD induced a decrease in the membrane potential of mitochondria ($\Delta\Psi$m), activated AMPK and inhibited the PI3K/Akt/mTOR Molecular pathway, induced mitophagy and autophagy in A549 cells, and subsequently caused mitochondrial-dependent apoptosis.

Cell cultures and reagents

Non-small cell lung cancer cell line A549(Shanghai Institute of Cell Biology, China) was cultured in RPMI medium 1640 (Life Technologies Corporation, Grand Island, NY, USA)added with 10 % fetal bovine serum (Gibco Corporation, Carlsbad, CA,USA), 1 mM glutamine 1.5 g/l sodium bicarbonate, and 1 mM sodium pyruvate at5 % CO $_2$. 37 °C. The Antibodies against Bcl-2,Bax,PARP-1,Mcl-1, Bcl-xL, Bcl-2, Bax,caspase-3,caspase-8, caspase-9, PARP, AMPK, p-AMPK,,Akt, p-Akt,,c-Jun,,p-c-Jun,,JNK,,ULK1,p-JNK,,mTOR,Beclin-1,LC3, p62 and GAPDH, DAPI, Tom20 were purchased from Cell Signaling Technology. cyclinA, cyclin B2,cyclin D1,cyclin D3, p21and Cdc2were purchased from Sigma-Aldrich. Analytical grade NCTD was obtained from Sigma-Aldrich (St. Louis, MO, USA). A stock solution of NCTD (50 mM) made in Dulbecco's modified Eagle's medium (DMEM, Invitrogen, Victoria, Australia) was prepared and stored at -20 ° C. As described previously [8], the 3- (4,5-dimethylthiazol-2-yl) -2,5-diphenyltetrazole bromide (MTT) method was used to detect the drug on cells Proliferation inhibition rate

Cell proliferation assay

MTT assay was used to evaluate the inhibition of cell proliferation. Cells were collected, adjusted to a cell concentration of 4.1 × 104 cells / ml, and 100 microliters were seeded in a 96-well plate. After 24 hours of incubation, NCTD with final concentrations of 0, 0.160, 0.312, 0.625, 1.25, 2.5, 5, 10, 20, 40, and 80 $\mu$M were added to each well. The group without NCTD treatment was the control group. Then continue to incubate for 72 hours, then add MTT solution (15.0 $\mu$l / well), and continue incubation for 4 hours in a 5% CO2 incubator. Remove the culture plate and add 150 $\mu$l of dimethyl sulfoxide (DMSO) to dissolve the purple precipitate. A 96-well plate was analyzed on an automatic microplate spectrophotometer (Thermo Molecular De-vices Co., United States) at a wavelength of 560 nm. Calculate each cell (A560 control cells-A560 treated cells) / A560 control cells x 100% cytostatic rate. The IC50 value is calculated by the logit equation. Experiments were performed in triplicate.Clone formation assay

Clone formation assay

The clone formation experiment was used to evaluate the effect of NCTD on the cloning ability of A549 cells. A549 cells were cultured in a 6-well microplate (1000 cells / well) in 2 ml of 10% fetal calf serum and 5% CO2 humidified atmosphere at 37 ° C overnight. The cells were then treated with the prepared drug working solution NCTD on a 6-well plate for 7 days, and then stained with 0.1% crystal violet (Sigma, St. Louis, Mo, USA) for 15 minutes. A digital camera (Canon, EOS350D, Tokyo, Japan) was used to obtain images of the number of colonies.

Cell apoptosis and death analysis

Flow cytometry (Beckman Coulter, Fullerton, California, USA) is used to distinguish the apoptotic period and quantity through different periods of apoptosis and different membrane permeability to dyes. In order to detect the apoptosis rate of the drug, the A549 cell density was adjusted to 2 × 105 cells / well in a 6-well plate, and the next day was exposed to final concentrations of 0, 5, 10, 20, and 40 $\mu$M NCTD. According to the manufacturer's protocol, Annexin V-FITC / PI (Becton Dickinson, USA) was used to perform the operation. The translocation of phosphatidy lserine to the cell surface is an indicator of early apoptotic cells. Therefore, Annexin V-positive and PI-negative cells were identified as apoptotic. Cell Quest software (FCM,

Becton Dickinson, USA) was used to determine the apoptotic rate.

Cell cycle analysis

In this study, propidium iodide (PI) (Becton Dickinson, USA) was used to stain nuclear DNA of tumor cells to identify cell cycle changes. A549 cells were cultured for 24 hours after exposure to the compound NCTD at concentrations of 0, 5, 10, 20, and 40 $\mu$M. Approximately 1 x 105 cells were collected and fixed in 70% ice-cold ethanol overnight. The fixed cells were incubated with 15 units / ml of RNase I and 100 $\mu$g / ml of propidium iodide for 15 minutes. Cellular DNA content was measured by flow cytometry. Data results were statistically analyzed by Cell Quest software (FCM, Becton Dickinson, USA).

Measurements of mitochondrial membrane potential ($\Delta\Psi$m)

JC-1 dye (Beyotime Co, Hangzhou, China) was used to detect changes in mitochondrial membrane potential ($\Delta\Psi$m) of tumor cells, and this dye indicated mitochondrial depolarization by changing the fluorescence intensity from high red to low green. A549 cells were seeded in 6-well plates, adjusted to a cell density of 1 $\times$ 105 cells / well, and exposed to compounds NCTD at final concentrations of 0, 5, 10, 20 and 40 $\mu$M for 24 hours. Collect the cells and test them on a flow cytometer or spread the cells on a glass slide. Images were acquired under a fluorescence microscope (DFC480; Leica Microsystems, Wetzlar, Germany)..

Western blot assays

After treating A549 cells with different concentrations of 0, 5, 10, 20, and 40 $\mu$MNCTD for 24 hours, the cells were collected, and the concentration of each group of proteins was measured after adding lysate for 30 minutes. The whole process was performed on ice. An equal amount of protein was calculated based on the protein concentration. The volume of each group was added to a 10% SDS-PAGE gel for electrophoresis, and then the protein was transferred to a PVDF membrane. Block the membrane with 5% skimmed milk powder / TBST for 100 minutes at room temperature, and then react with the appropriate antibodies to be tested for diluted primary antibodies Bcl-2, Bcl-xL, Mcl-1, caspas-3 / 8/8, PARP , AMPK, p-AMPK, pc-Jun, p-Akt, Akt, c-Jun, JNK, ULK1, p-JNK, mTOR, Beclin-1, LC3, p62, cyclinA, cyclinB2, cyclinD1, cyclinD3, p21, Cdc2 And the internal reference GAPDH (diluted 1: 1000 in blocking buffer) at 4 ° C overnight, remove the membrane from the primary antibody the next day, wash 3 times in TBST for 10 minutes each time, and then peroxidize with horseradish The enzyme-conjugated secondary antibody was incubated for 1 hour at room temperature. Wash the photos before the method, add the developer Super Signal west pico (Thermo) together into the luminometer, and visualize the protein.

Immunocytochemistry

Log-grown A549 cells were seeded on sterile coverslips, and the cells were treated with two concentrations of PP-22 (0 or 4 $\mu$M) for 24 hours. Cells on the coverslips were fixed with 4% paraformaldehyde (PFA) for 10 minutes. Cell washed 3 times with cold PBS every 3 minutes, permeabilize cells with 300 $\mu$l of 0.25% Triton-X 100 at 4 ° C for 16 minutes, and treat cells with 4% bovine serum albumin soluble backgrou binding for 1.5 hours, and continuously extend with LC3 antibody (1: 100) at 4 ° C. Cell is washed 3 times with PBST (PBS with Tween-20), then keep at room temperature with Alexa Fluor 488 labeled goat anti-rabbit IgG for 1.5 hours temperature according to manufacturer's instructions. Then, the cell stained with 4 ', 6-dimidyl-2-phenylindole, dihydrochloride (DAPI) (300 $\mu$l / group for 10 minutes), and washed 3 times with PBST again. Cell detected under a confocal microscope (Olympus, Japan).

Statistical analysis

Data are expressed as the mean $\pm$ standard error of the mean and were analyzed using Graph Pad Prism Version 7 (Graph Pad Software Inc., San Diego, CA) and SPSS 20.0 software. All experiments were designed in triplicate

Results

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

To determine whether NCTD has a cytotoxic effect on human cancer cells, we assessed the viability of human cancer cell lines (non-small lung cancer cell line A549, breast cancer MCF-7 cell line , hepatocellular carcinoma BEL-7402 cell line, nasopharyngeal carcinoma CNE-2 cell line) and human hepatic cell line LO2. Cells were exposed to different concentrations of NCTD for 72 h as revealed in **Fig. 1B.** NCTD showed significant proliferation inhibition and cytotoxic effects on these cancer cell lines, and showed moderate cytotoxic effects on normal LO2 cells. These data indicate that NCTD strongly inhibits tumor cell proliferation in a dose- and time-dependent manner. After 72 h of cell exposure, NCTD inhibited A549 cell activity in a dose-dependent manner with an IC50 value of 13.1 $\mu$M (Table 1). In addition, the clone formation test showed that NCTD significantly inhibited the cloning ability of A549 cells (Figure 1C).

fig. 1

table 1

The effect of NCTD on the A549 cells cell cycle progression

Growth inhibition is often associated with cell cycle progression. Therefore, the cycle distribution of A549 cells after exposure to NCTD was measured by propidium iodide staining. As shown in Figure 2A, as the concentration of NCTD treatment increased, cell accumulation in the G2 / M phase increased, with a gradual decrease in the G1 population. The above results indicate that the cell cycle is blocked in the G2 / M phase.

The complex of cyclin and CDKs plays an important role in regulating the progress of the cell cycle, so the expression of cycle-related proteins was further tested by western blot method. After NCTD treatment of cells, the expressions of cyclin D3, E2, G1, and S phases were significantly reduced, while the expression of cyclin B1, cyclin A, and G2 / M phase-related proteins showed an upward trend (Figure 2C). . Moreover, p21 expression was also found to increase, while cdc2 expression was reduced (Figure 2B). The activity of the M phase promoting factor cdc2 / cyclinB complex plays a crucial role in the G2 to M phases. Our results indicate that despite the increased expression of cyclin A and cyclin B1 (G2-phase related proteins), p21 can inhibit the activity of the cdc2 / cyclin B complex. Therefore, we can infer that NCTD induces G2 phase cell cycle arrest by increasing the expression of p21.

fig. 2

NCTD induced apoptosis and reduced the mitochondrial membrane potential

In order to further study the mechanism of NCTD-induced cell proliferation inhibition, flow cytometry was used to analyze the changes in the apoptosis rate of cells after exposure to different concentrations of the compound NCTD. As shown in Figures 3A and 3C, after NCTD treatment of tumor cells, the percentage of A549 cells' apoptotic rate increased from 4.0% to 40.1%, indicating that the rate of A549 cell apoptosis increased with the increase of NCTD

Depolarization of mitochondrial membrane potential ($\Delta\Psi$m) is the key to cell death. Therefore, we used JC-1 staining to examine changes in mitochondrial membrane potential. As shown in FIG. 3B, as the NCTD concentration increased, $\Delta$ 降低 m in A549 cells gradually decreased. As shown in FIG. 3D, compared with the control group, cells treated with 20 $\mu$M and 40 $\mu$M NCTD for 24 hours both showed high green and low orange fluorescence under the fluorescence microscope, while the control group detected high green and high orange fluorescence.

fig.3.

**NCTD induced cells apotosis through Bcl-2 family and caspase family**

In addition we investigated the expression levels of Bcl-2 and caspase families in NCTD-induced apoptosis. As show in Figure 4A, results revealed that Bcl-2 and Mcl-1 were markedly decreased after exposed to different concentrations of NCTD for 24 h. whereas the expressions levels of Bax and Bcl-xl, did not change significantly. Furthermore As shown in Figure 4B, the protein expressions levels caspase-3, caspase-9 and

PARP decreased, however pro-caspase-8 has not significantly changed. in Collectively, NCTD induced A549 cell apoptosis via the mitochondrial signaling pathway.

fig.4

NCTD-induced autophagy and mitophagy

LC3 is considered to be the initiation factor of autophagy-specific markers. Our research shows that, after tumor cells are exposed to NCTD, LC3I transforms to LC3II, and the expression level of SQSTM1 / P62p62 decreases. As shown in Figure 5A. This is the same as we observed the aggregation of LC3 under a confocal microscope. The accumulation of punctate LC3 in the cytoplasm of A549 cells (as shown in Figure 5C). The above data indicate that NCTD induced autophagy in A549 cells. Mitochondrial depolarization is a crucial early event in mitochondria [6]. We found that NCTD caused a decrease in mitochondrial membrane potential ($\Delta\Psi$m) (fig3). TOM20 protein is a mitochondrial outer membrane protein, so its reduced expression can reflect mitochondrial phagocytosis [9]. To further investigate whether mitochondrial phagocytosis occurs in cells treated with NCTD, we used immunoblotting to treat A549 at NCTD The expression of TOM20 after cell decline was shown in Figure 5B; a characteristic aggregation of TOM20 around the nucleus was observed (indicating mitochondria) (Figure 5D), These data suggest that NCTD induced cell phagocytosis in A549 cells

fig.5.

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Effect of NCTD on the expression of the Akt/mTOR pathway and the JNK/c-jun pathway

PI3K / Akt / mTOR, AMPK / mTOR and JNK play important regulatory functions in various cell life activities, such as apoptosis, cell cycle rhythm, differentiation, growth, etc. We examined the expression of p-Akt, p-AMPK, mTOR, p-JNK and p-c-jun in NCTD-affected A549 cells by Western blot. As shown in Figure 6A, the NCTD concentration and the expression of p-Akt and mTOR showed a negative correlation. However, it is positively correlated with p-AMPK and ULK1. In addition, the expression of p-JNK and its downstream protein pc-jun gradually increased with increasing NCTD concentration in treated cells (Figure 6B). The above data indicate that the activation and inhibition of the AMPK / mTOR and JNK / c-jun pathways are involved in the Akt / mTOR signal transduction pathway and are related to apoptosis and autophagy of A549 cells.

Fig6.

Fig7

Discussion

More and more studies have shown that NCTD can induce apoptosis in a variety of tumor cells. However, so far, whether NCTD has an effect on non-small cell lung cancer (NSCLC) cells is unknown. In this study, we used different concentrations of NCTD to observe the proliferation of A549 cells and explore its molecular mechanism. MTT analysis showed that the proliferation ability of A549 cells decreased with the increase of NCTD concentration. The colony forming ability experiments showed that the increase in NCTD concentration was inversely related to the number of cell clones. These data indicate that NCTD has an inhibitory effect on cell proliferation and activity (Figure 1B, 1C). The cell cycle plays an important role in the development of malignant tumors, and cycle changes are a major feature of malignant tumors [10]. Previous studies have shown that tumor cell development may be related to changes in the expression of cell cycle-related regulatory genes (including cyclin-dependent cyclin-related genes), thereby blocking cells in the G2 / M phase (CDKIs; such as p21) [11,12]. Then we used flow cytometry to find that as the drug concentration increased, the proportion of cells in the G2 phase of the tumor cells also increased. Figure 2A, which shows that NCTD downregulates cyclin D3 and cyclin E2 and upregulates cyclin A and cyclin B (**Figure 2C** ), the results are consistent. These data indicate that NCTD can inhibit the proliferation of A549 cells by blocking the G2 phase. p21 is a member of the Cip / Kip family and can regulate CDK and

cyclin, thereby affecting the transition from G2 to M phase. Our findings (Figure 2B, C), even though cyclin A and cyclin B (G2 phase-related proteins) increase, p21 still inhibits the activity of the cdc2 / cyclin B complex, so we conclude that NCTD increases the expression of p21 Inducing G2 cell cycle arrest, Akt may also inhibit p21 expression through its phosphorylation and MDM2 activation and subsequent p53-mediated down-regulation of p21 transcription. Our results indicate that the expression of p-AMPK is increased and the expression of p-Akt is decreased (Figure 6A), indicating that NCTD activates AMPK, inhibits Akt, up-regulates p21 expression and inhibits the active B complex of cdc2 / cyclin, thereby stopping the cell cycle. G2 / M.

Apoptosis is the main form of tumor cell death. In our study, flow cytometry analysis of NCTD-treated tumor cells was found to have a positive correlation between the apoptotic rate and NCTD concentration (**Figure 3A, C** ). Apoptosis is associated with cytochrome c release, caspase-3 activation, and PARP cleavage [13]. Enhanced permeability of the outer mitochondrial membrane, reduced mitochondrial membrane potential, mitochondria release pro-apoptotic molecules such as cytochrome C to the cytoplasm, thereby activating caspase-9, causing a cascade reaction leading to cell death [14,15]. In this study, we found that NCTD reduced the $\Delta\Psi$m point. (Figure 3B, D). Bcl-2 and Mcl-1 decreased in the Bcl-2 family, while Bax and Bcl-xL remained unchanged, resulting in an increase in the ratio of Bax / Bcl-2 and Bax / Mcl-1 (Figure 4A). In the caspase family into caspase-3, the activation of caspase9 (**Figure 4B** ) activates caspase-3 to cleave PARP and cause changes in morphology and biological apoptosis. Because the caspase-8 protein changes are not obvious, we speculate that NCTD-induced A549 cell apoptosis does not involve an exogenous apoptotic pathway **(Figure 4B** ).

Autophagy mainly protects cells from external stimuli and plays a role in regulating and controlling the internal environment of the cell [16], but excessive autophagy consumes components in the cell and causes cell death[17]. Previous studies have shown broad prospects for the treatment of malignant tumors through autophagy. In this study, we demonstrated that NCTD treatment can increase the conversion of LC3-I to LC3-II, reduce the expression of p62 (Figure 5A), and increase the spotted LC3 in the A549 cytoplasm (Figure 5C). It is shown that NCTD induces autophagy in A549 cells.Mitochondria is a mitochondrial recovery process that mainly promotes cell survival, but may also lead to cell death under conditions of excessive injury. Mitochondrial depolarization is an early event in which physiological function is affected in mitochondria [18]. The function of mitochondrial phagocytosis has been studied and is thought to be an early manifestation of cellular autophagy. Mitochondrial autophagy induces mitochondrial phagocytosis [19]. TOM20 protein is a mitochondrial outer membrane marker protein, so changes in its expression level can reflect mitochondrial autophagy status [20]. It was found in the study that NCTD induced a significant decrease in $\Delta\Psi$m in A549 cells (Figure 3B, 3D). After NCTD treatment of cells, the expression of TOM20 protein was reduced (Figure 5B) and characteristic aggregation of mitochondria was triggered. (Figure 5D). The above data indicates that NCTD induced mitochondrial phagocytosis in A549 cells.

Previous studies have shown that the Akt / mTOR signaling pathway plays an important role in cell proliferation, and the activation of this pathway is related to the occurrence and development of various malignancies[20]. This study found that the antitumor drug NCTD induces early autophagy and late apoptosis of tumor cells through the Akt / mTOR signaling pathway [21]. mTOR is a key factor in autophagy activation.[22]. The upstream mTOR signaling pathway plays an important role in cell growth and cell cycle, mainly through the PI3K / Akt / mTOR pathway and the independent PI3K / Akt pathway. Akt belongs to the serine / threonine protein kinase subfamily and is a kinase upstream of AMPK. AKT inhibits AMPK and activates mTOR through the AMPK-TSC-MTOR pathway [23]. Previous studies have shown that NCTD can activate AMPK in mammalian cells[24]. AMPK can induce autophagy directly through the ULK1 protein or indirectly through inhibition of the mTOR signaling pathway [25]. In this study, NCTD reduced the expression of p-Akt, increased the expression of p-AMPK and ULK1, and suppressed the expression of mTOR, suggesting that NCTD promoted autophagy in A549 cells (Figure 6A). C-jun-N-terminal kinase (JNK) is a mitogen-activated protein kinase family member that can be activated by a variety of external factors, such as radiation, bacteria, drugs, hypoxia, and endoplasmic reticulum stress. JNK signal transduction in cell proliferation, differentiation, apoptosis, ROS accumulation [26]. JNK-mediated 14-3-3

protein phosphorylation promotes its dissociation from Bax and translocation to mitochondria, leading to Cell death. Phosphorylation of JNK-mediated Ser70 of Bcl-2, Thr47 and Thr115 of Bcl-xL, and Ser121 and Thr163 of Mcl-1 can inactivate these anti-apoptotic proteins in response to cellular stress. Similarly, JNK-mediated phosphorylation of Ser128 in Bad, Ser65 in Bim, Thr56 in BimL, and Thr67 in Bax promote these proapoptot-ic proteins[26]. In this study, we found that NCTD activates JNK and downstream c-jun proteins, promoting apoptosis and autophagy (Figure 6B).

As shown in Figure 7, NCTD treatment activated Akt, then up-regulated p21 and inhibited the activity of cdc2 / cyclin B, and finally induced cell cycle arrest in the G2 / M phase. Inhibits p-Akt, downregulates mTOR, up-regulates ULK1, and promotes autophagy. AMPK activation also inhibits mTOR expression and increases ULK1, thereby promoting cellular autophagy. On the other hand, inhibition of Akt and activation of JNK and AMPK together regulate the inhibition or activation of members of the Bcl-2 family. The negative regulation of Bcl-2 and Mcl-1 and the reduction of mitochondrial membrane potential induce mitochondrial autophagy. The above changes cause the activation of the caspase family, leading to the cleavage of PARP and ultimately the apoptosis of A549 cells. Mitochondrial activation is also involved in autophagy and apoptosis induced by drug action on A549 cells. In general, NCTD can induce tumor cell autophagy and apoptosis by regulating the Bcl-2 family, inducing mitochondrial dysfunction, activating the caspase family, inhibiting the PI3K / Akt / mTOR pathway, and activating the AMPK / ULK1 and JNK signaling pathways ,NCTD causes cytotoxicity to trigger A549 cell apoptosis

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**Conflict of interest**

The authors declare no conflicts of interest concerning this article.

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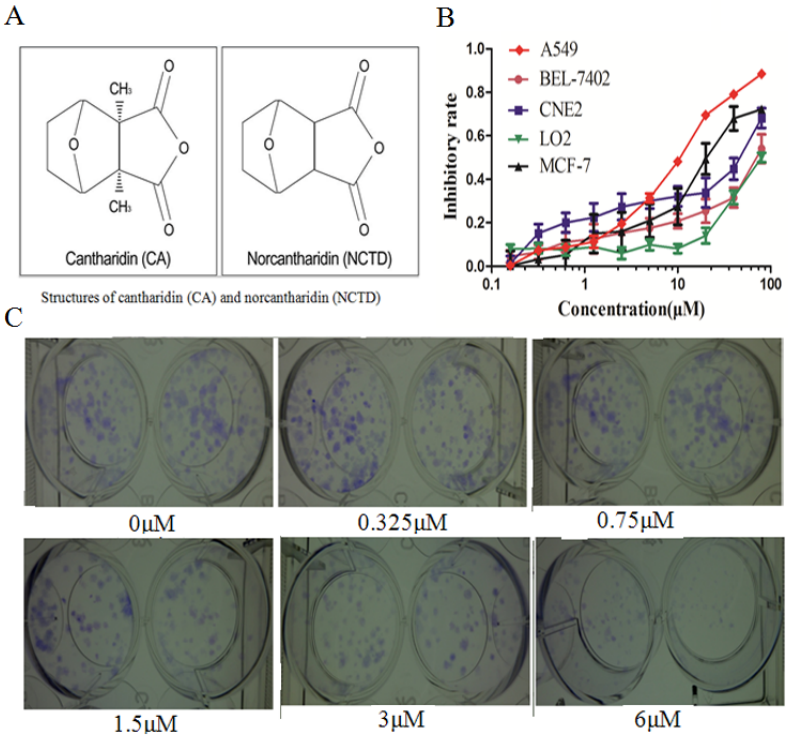


**Figure 1.**

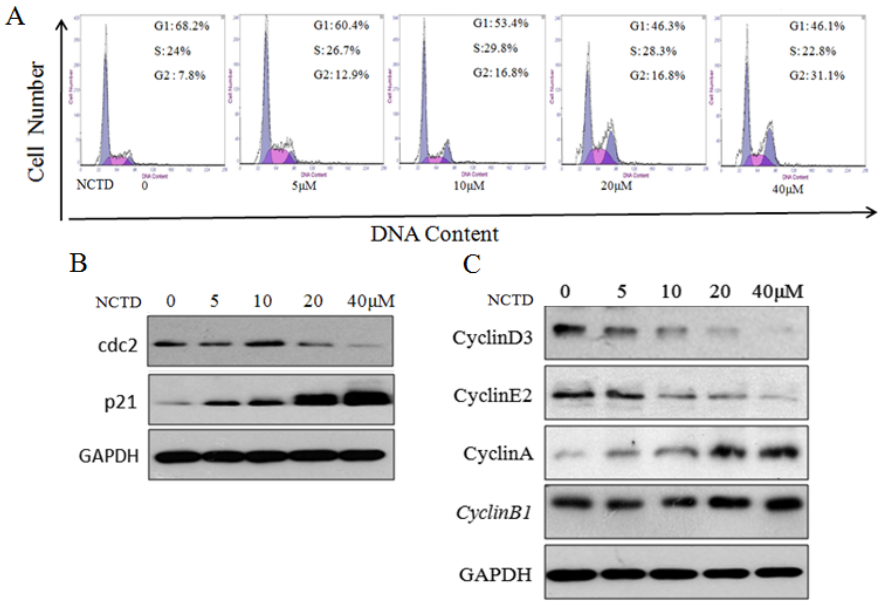


Figure 2.

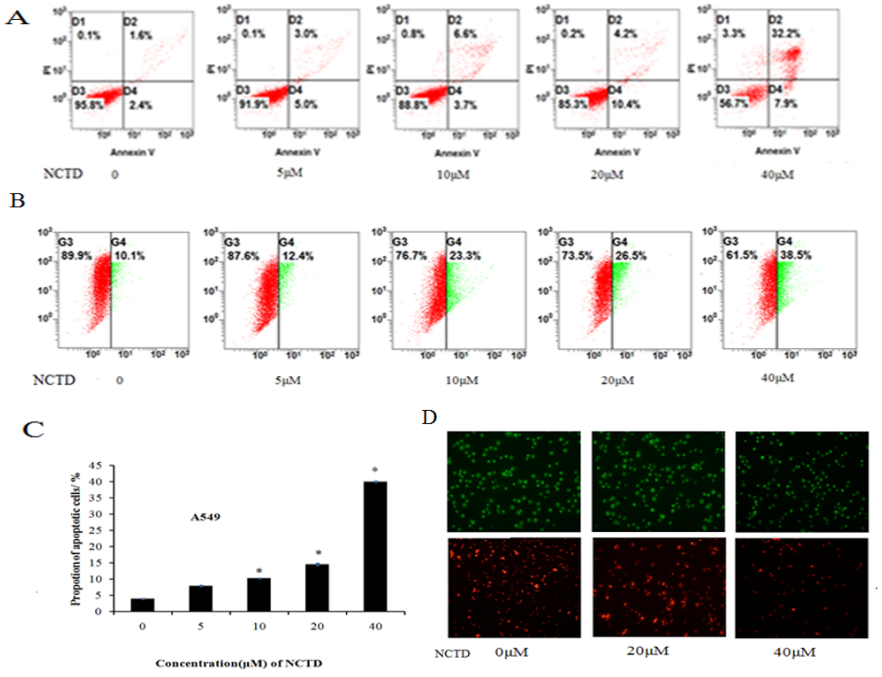


Figure 3.

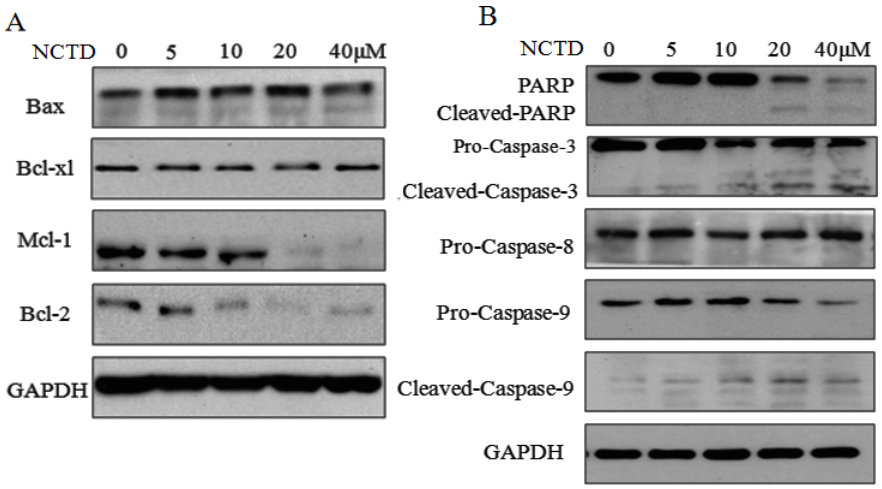


Figure 4.

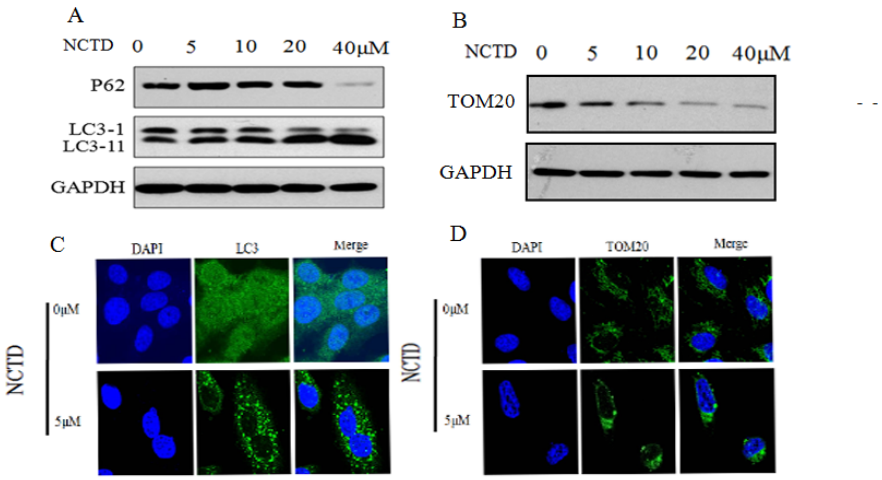


Figure 5.

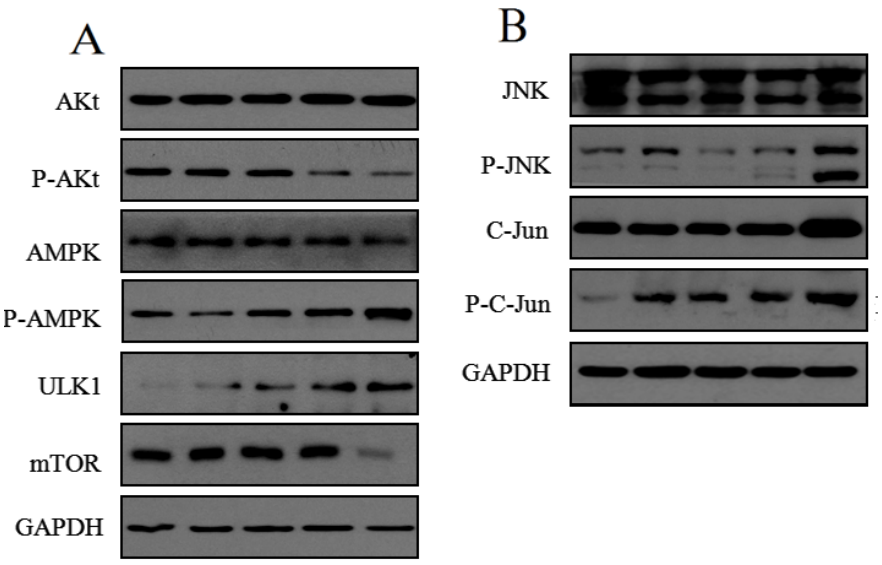


Figure 6.

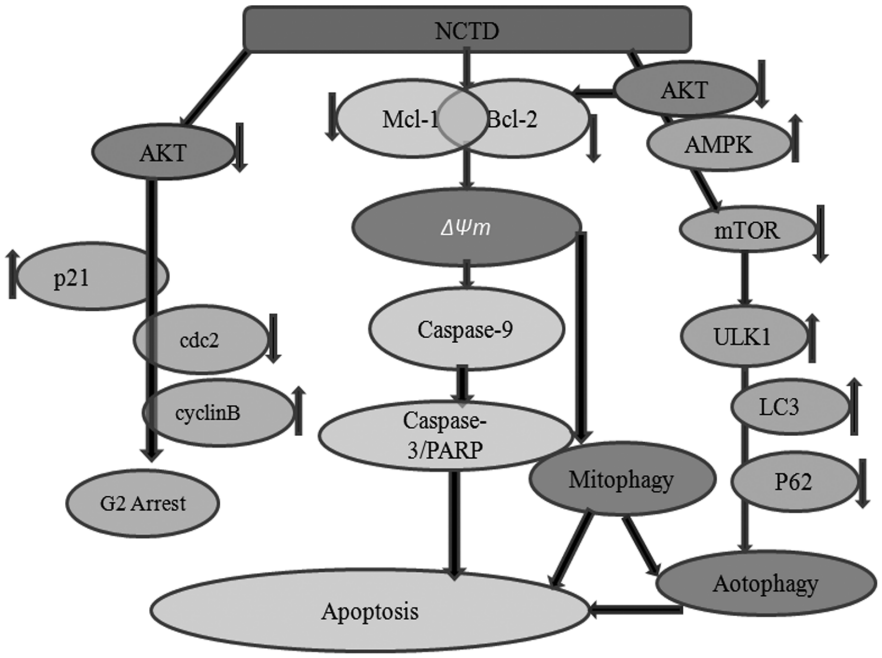


Figure 7.