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Original research paper

Norcantharidin induces G2/M arrest and apoptosis *via* activation of ERK and JNK, but not p38 signaling in human renal cell carcinoma ACHN cells

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Renal cell carcinoma (RCC) is generally acknowledged as the most resistant primary malignancy unresponsive to conventional radiotherapy and chemotherapy treatments. Norcantharidin (NCTD), a therapeutic compound derived from medicinal plants, has been shown to trigger apoptosis, as well as antimetastatic and antioxidant activities in several tumor cells. However, NCTD's mechanism of antitumor activity in the RCC cell line remains unclear. In this study, we report that NCTD led to a time- and dose-dependent inhibition of cell proliferation. It had also markedly induced apoptosis and G2/M phase cell cycle arrest in a dose-dependent manner by decreasing the expressions of pro-caspase-3, pro-caspase-9, cyclin B1, and pCDC25C while increasing active caspase-3, cleaved-PARP, P21, and pCDC2 levels. Interestingly, NCTD treatment provoked the phosphorylation of extracellular-regulated protein kinase (ERK) and c-Jun-N-terminal kinase (JNK), but not of p38 MAPK. Moreover, SCH772984 and SP600125, ERK and JNK inhibitors, respectively, could partially abolish NCTD-induced apoptosis and G2/M phase cell cycle arrest. Collectively, these findings suggest that NCTD might activate JNK and ERK signaling pathways, consequently inducing apoptosis and G2/M arrest through the modulation of related proteins. This study provided evidence that NCTD is a promising therapeutic drug for the treatment of RCC.

Keywords: norcantharidin, renal cancer cells, ERK, JNK, p38 MAPK, apoptosis, cell cycle arrest

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Renal cell carcinoma (RCC) is the most commonly occurring malignancy in kidneys, specifically in the proximal renal tubular epithelial cells (1). Due to frequent metastasis, only one-third of RCCs can be effectively managed by resection, chemotherapy and radiotherapy (2, 3). Hence, new molecular targets should be identified, and more effective and less toxic drugs need to be developed for RCC treatment.

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Cantharidin is a traditional Chinese medicine extracted from the dried body of blister beetle (*Mylabris phalerata,* Pallas) known for its antitumor properties. Its properties can be traced back to 2000 years ago since it has been used for a long time. However, it causes side effects like leukocytosis and the stimulation of the urinary system (4–6).

For the sake of finding a cantharidin analogue with fewer side effects, norcantharidin (NCTD), a demethylated form of cantharidin, was prepared. NCTD exhibited stronger antitumor activity and caused fewer metabolic disturbances and nephrotoxicity than cantharidin because it could prevent hydroperoxide production in macrophages, and maintain tissue homeostasis in the kidney (7–9). Furthermore, NCTD inhibited the proliferation of metastatic, angiogenic, antioxidant, and antitubulointerstitial fibrosis, and also induced apoptosis and cell cycle arrest (10–13). The antitumor activity of NCTD has been observed in various types of cancer cells, such as prostate, breast, melanoma, and hepatocellular carcinomas (13–16). Nevertheless, only a few reports exist in the literature regarding the cytotoxic effects of NCTD on RCC cells.

Certain anticancer chemotherapeutic drugs exert their antitumor activities *via* the induction of cell cycle arrest and apoptosis (17). During the transition, a series of cyclins and cyclin-dependent kinases (CDKs) accurately regulate the progression of the cell cycle. Therefore, the abnormal expression of such proteins may lead to cell cycle arrest or cell death (18, 19). In addition to blocking cell cycle progression, chemotherapeutic drugs often cause apoptosis, a process of programmed cell death that occurs in response to toxicants (20, 21). Several biomarkers, such as Bcl-2 family and caspase family proteins, could be regarded as the determinants of apoptosis (22–24). Among these biomarkers, caspase-9 is responsible for activating other caspase members, while caspase-3 acts as the final apoptotic executive molecule (25, 26).

Mitogen-activated protein kinases (MAPKs) play an important role in the process of intracellular signal transduction by managing cellular responses to a diverse array of stimuli that regulate cell functions, including cell proliferation, differentiation, mitosis and apoptosis (27, 28). Extracellular-regulated protein kinase (ERK), c-Jun-N-terminal kinase (JNK), and p38 MAPK are key members of the MAPK family that are associated with the induction of cell cycle arrest and apoptosis (28, 29). However, it is unclear whether the MAPK signaling pathway participates in NCTD-induced cell apoptosis.

In this study, we observed the antitumor activity of NCTD with respect to RCC cells. We further explored the role of MAPK signaling in NCTD-caused cell cycle arrest and apoptosis.

EXPERIMENTAL

Reagents

A 100 mmol L^{-1} standard NCTD solution (≥ 98 %) was prepared by dissolving NCTD powder, purchased from Solarbio (Beijing, China), in dimethylsulfoxide (DMSO), and it was stored at 4 °C. Working solutions were freshly prepared from the standard solution and used in cell culture media. Fetal bovine serum (FBS), penicillin, streptomycin, and MTT were obtained from Sigma-Aldrich (USA).

Cell cultures

The ACHN human renal carcinoma cell lines were purchased from the Cell Bank of the Chinese Academy of Sciences (China). ACHN cells were cultured in DMEM (Hyclone, USA). All cells were incubated at 37 °C in 5 % CO_2 after supplementing the culture media with 10 % heat-inactivated fetal bovine serum (Hyclone, NZ), 100 U mL^{-1} streptomycin, and 100 mg mL^{-1} penicillin (Hyclone, USA).

MTT assays

Cell toxicity experiments were performed using the CellTiter96® AQueous One Solution Cell Proliferation Assay (Promega, Madison, Wisconsin, USA) according to the manufacturer's protocol. Briefly, cells were plated in 96-well plates at a density of 2000 cells/well. 20 µL of CellTiter 96® Aqueous One Solution Reagent were added to each well after culturing for 24, 48, 72, and 96 h at 37 °C. Absorbance values were measured at 492 nm using a spectrophotometer.

Flow cytometric analysis

The cell cycle detection kit (MultiSciences, China) and the AnnexinV/propidium iodide (PI) apoptosis detection kit (MultiSciences) were used to determine the effect of NCTD on cell cycle distribution and the percentage of apoptotic cells. For cell cycle analyses, ACHN cells were cultured in 6-well plates then trypsinized and fixed in 100 % ethanol at –20 °C for 24 h. The cell pellets were then stained with PI and simultaneously treated with RNase for 30 min at room temperature before being analyzed using a BD FACScan laser flow cytometer. The obtained data were analyzed using MODFIT and CellQuest software. Meanwhile, for cell apoptosis analysis, 1.0×10^6 cells were harvested and washed with cold PBS then resuspended in 1.0 mL binding buffer containing 5 µL Annexin V-FITC and 1 µL PI. These cells were detected immediately after supravital staining. Cells distributed in Annexin V positive and PI negative stand for the early stage of apoptosis, and late stages of apoptosis were both Annexin V and PI positive (30).

Western blot analysis

For Western blot analyses, cells were lysed with RIPA buffer (Solarbio, China) containing 1 % PMSF protease inhibitor (Solarbio). Total protein concentrations were measured using the BCA protein assay kit (Beyotime, China). Total protein (35 µg) samples were loaded and separated using 12 % SDS-PAGE, transferred to PVDF membranes, blocked with 5 % non-fat dry milk, and then incubated overnight with specific primary antibodies (Supplementary Material 1) at 4 °C. Thereafter, the blots were washed with TBST, incubated with horseradish peroxidase-labeled secondary antibodies (Boster, China), and visualized using an enhanced chemiluminescence reagent (Clinx, China). The gray value of each blot was detected by a gel imaging system (Tanon, China).

Inhibitor treatment

To investigate the effect of MAPK inhibitors on NCTD-induced apoptosis and cell cycle arrest, cells were pretreated for 1 h with 1 µmol L^{-1} SCH772984 (ERK specific Inhibi-

tor) (Selleckchem, USA) or 5 μmol L^{-1} SP600125 (JNK specific inhibitor) (Selleckchem) or 5 μmol L^{-1} SB203580 (p38 MAPK specific inhibitor) (Selleckchem) before the addition of NCTD.

Statistical analysis

All data were shown as means ± SD of three independent experiments. Data were analyzed using SPSS software, version 18.0 (SPSS, Inc., USA). Statistically significant differences between the two groups were determined by Student's *t*-test, three or more than three groups were calculated using ANOVA (post hoc test: Dunnett's, multiple comparisons), $p < 0.05$ was considered as statistically significant.

RESULTS AND DISCUSSION

Effective antitumor drugs are urgently needed to improve the prognosis of renal cancer. Recently, it has been reported that NCTD, a highly cytotoxic compound, is a promising anticancer drug, particularly considering fewer side effects and stronger antitumor activities associated with its use (8, 9). The aim of this study is to investigate the effect of NCTD on RCC cells and elucidate the underlying mechanisms.

Several studies have proven that NCTD constrains the proliferation of various types of tumor cells (8, 13–15). The structure of NCTD is presented in Fig. 1a. To explore the role of NCTD in cell growth, ACHN cells were treated with NCTD solutions at concentrations of 0, 10, 50, 100, 500, or 800 μmol L^{-1} for 0, 24, 48, 72, or 96 h. MTT assay results showed that NCTD substantially inhibits ACHN cell growth in a time- and dose-dependent manner (Fig. 1b). The concentration resulting in 50 % inhibition of NCTD after 24, 48, 72 and 96 h treatment was shown in Table 1. In our preliminary experiment, we discovered that a low concentration of NCTD (10 μmol L^{-1}) could modify the cell cycle and early apoptosis, but it didn't significantly inhibit cell proliferation. Therefore, the concentrations of 10, 100 and 200 μmol L^{-1} were applied for further experiments. Exposure of ACHN cells to NCTD for 24 h induces G2/M phase cell cycle arrest (Fig. 2a). This effect becomes more prominent as the concentration of NCTD increases between 0 and 200 μmol L^{-1}. Generally, cell cycle

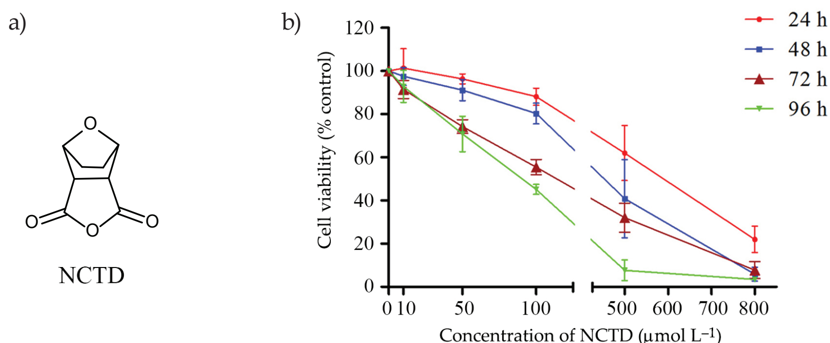

Fig. 1. NCTD inhibited cell proliferation in ACHN cells. a) Chemical structure of NCTD, b) cell proliferation of ACHN measured by MTT assay.

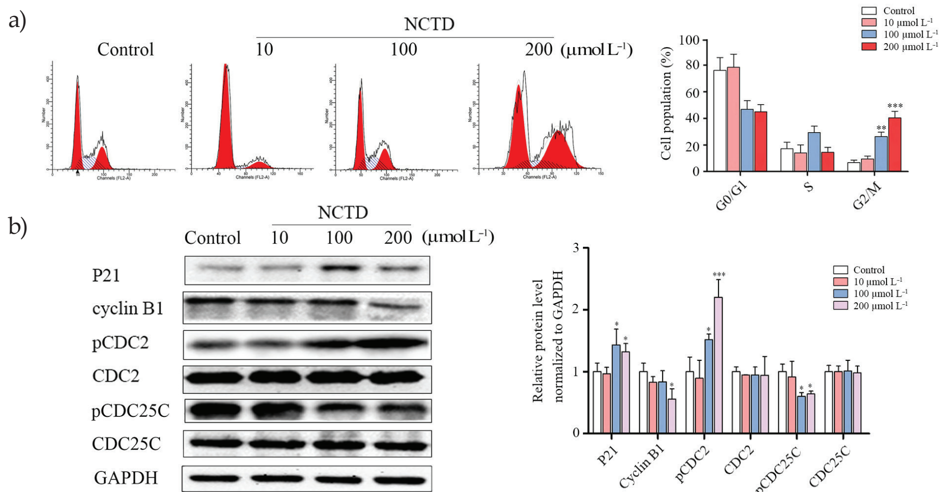

Fig. 2. NCTD induced G2/M phase cell cycle arrest and altered cell cycle-regulatory proteins expressions in ACHN cells. a) Cell cycle distribution and b) related regulatory proteins monitored after treated with 0, 10, 100, and 200 μmol L^{-1} NCTD for 24 h. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ *vs*. control group.

transmission requires the coordination of several key proteins. Both cyclin B1 expression and dephosphorylation of pCDC2 are needed to activate the CDC2/cylcin B1 complex and cross the G2/M checkpoint to enter mitosis (31, 32). The CDC2/cylcin B1 complex may be repressed by P21; thus, the accumulation of P21 in the nuclei near the G2/M boundary could result in the G2/M phase block (33). Meanwhile, the translocation of CDC25C protein phosphatase into cellular nuclei catalyzes the dephosphorylation of CDC2, which in turn activates the cyclin B1/CDC2 complexes at the Tyr 15 site (34, 35). To further address the induction of G2/M phase arrest by NCTD in RCC cells, the expression levels of P21, cyclin B1, pCDC2, CDC2, pCDC25C, and CDC25C were monitored using western blot analyses. The results depicted in Fig. 2b indicate that NCTD treatment induces a concentration-dependent decrease in cyclin B1 and pCDC25C expressions, whereas it increases the levels of P21 and pCDC2. Such alternations in protein expressions deactivate the cyclin B1/CDC2 complexes, thereby blocking the G2/M phase progression. Moreover, defects in G2/M checkpoint arrests may allow damaged cells to enter mitosis and undergo apoptosis, an active form of cell death in response to toxicants.

Apoptosis is associated with the release of cytochrome c and the increase in cell permeability. It may be instigated by the caspase family protein cleavage of PARP (36). As shown in Fig. 3a, NCTD treatment causes a significant dose-dependent increase in total apoptosis. Meanwhile, western blot results indicate that an increase in NCTD concentration leads to decreased pro-caspase-3/9 levels while increasing the levels of active caspase-3/9 and cleaved-PARP (Fig. 3b).

The mechanism of NCTD-induced cell apoptosis was further investigated by assessing the role of ERK, JNK and p38, three major members of MAPKs that can be activated by upstream dual-specificity kinases. The activation of the ERK cascade is primarily involved in cell transformation, differentiation and survival, whereas JNK and p38 cascades are

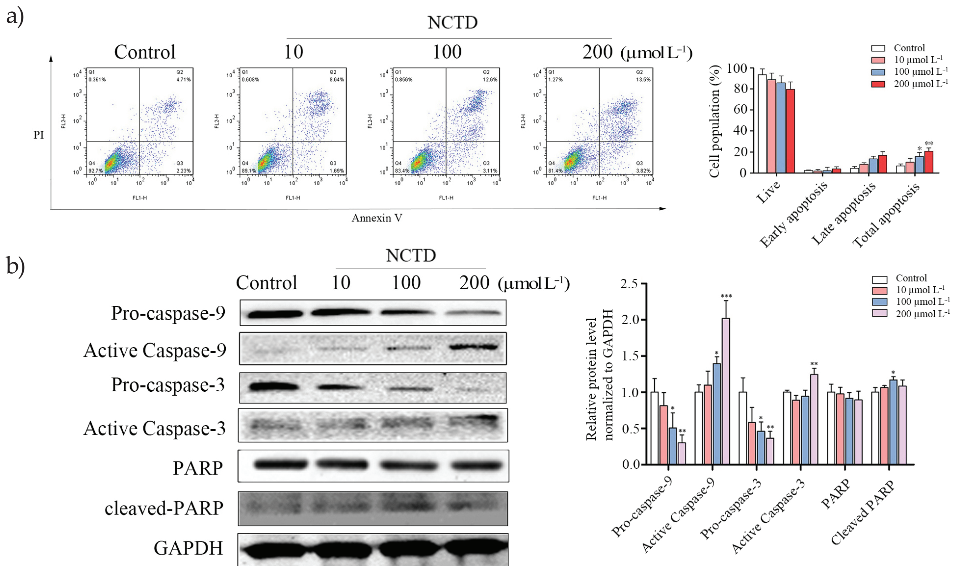

Fig. 3. NCTD induced cell apoptosis in ACHN cells. After cells were treated with 0, 10, 100, and 200 µmol L^{-1} NCTD for 24 h, AnnexinV/PI and Western blot were used to detect: a) apoptosis and b) its related proteins. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ *vs.* control group.

activated in response to physiologic, osmotic or genotoxic stress, endotoxins, pro-inflammatory cytokines, and UV exposure (4, 37). Han *et al.* report that activation of the JNK/c-Jun pathway by NCTD could accelerate mitochondrial cytochrome c release into the cytoplasm and reduce Bcl-2 and Mcl-1 expression levels, leading to caspase activation (38). Chen *et al.* show that NCTD-induced apoptosis was accompanied by elevated phosphorylated ERK and JNK levels and activity; however, it did not have any appreciable effect on p38 MAPK (39). Similar results were observed in this study, wherein we found that NCTD-

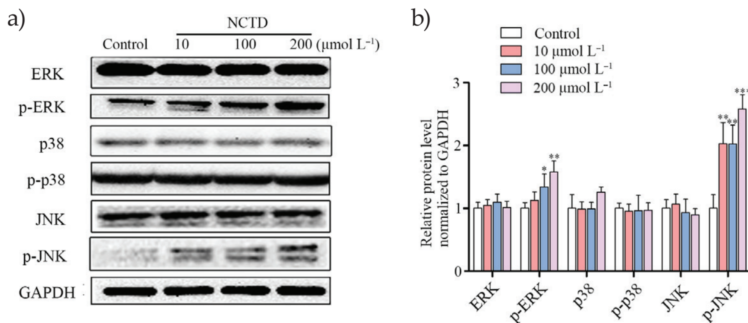

Fig. 4. NCTD induced the activation of ERK and JNK. Cells treated with 0, 10, 100, and 200 µmol L^{-1} NCTD for 24 h were subjected to Western blot assay.

treated RCC cells exhibit increased p-ERK and p-JNK levels, whereas those of p-p38 and p38 remain unchanged. The results indicate that NCTD activates JNK and ERK, but not p38, MAPK proteins in ACHN cells, in vitro (Fig. 4). Moreover, we show that the p38 inhibitor (SB203580) fails to attenuate the inhibitory effect induced by NCTD (data not shown), indicating that p38MAPK is not responsible for NCTD-inhibited cell viability.

To further understand the connection between JNK and ERK signaling pathways and NCTD-induced G2 phase arrest and apoptosis, the involvement of the two pathways was tested using their specific inhibitors of SP600125 and SCH772984. As shown in Fig. 5a,b, the proportion of G2/M phase cells in SP600125 pretreated NCTD group was significantly

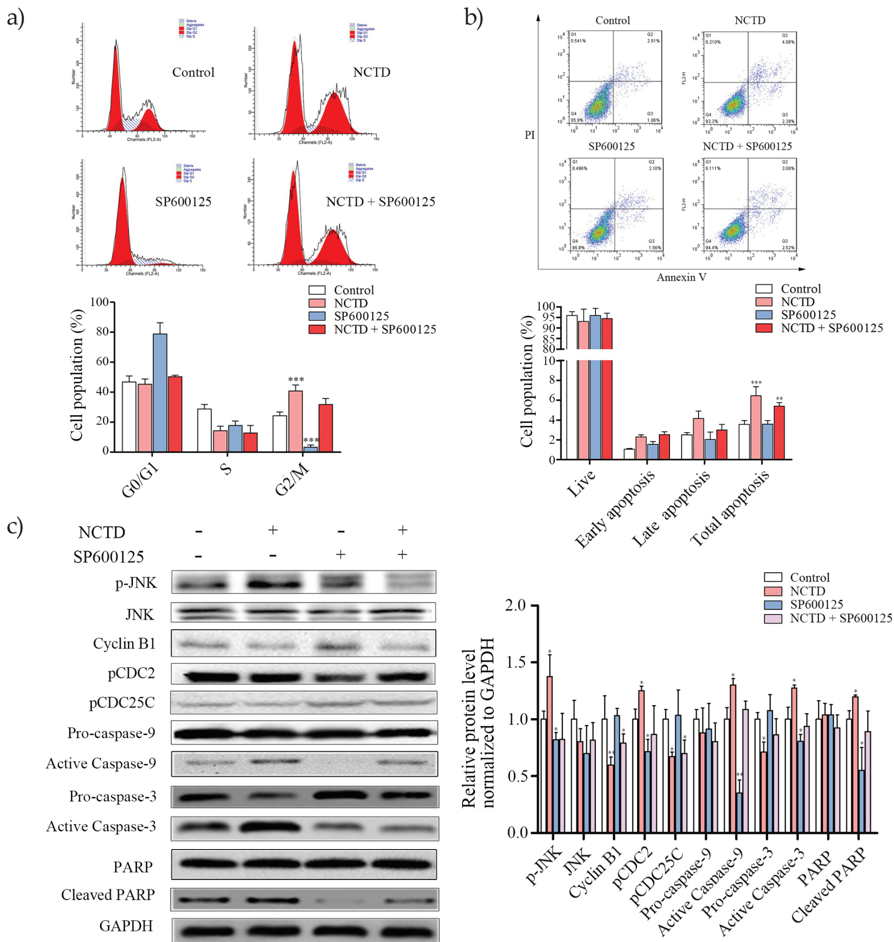

Fig. 5. SP600125 attenuate NCTD-induced G2/M arrest and apoptosis. RCC cell lines were pretreated with 5 µmol L^{-1} SP600125 before being exposed to NTCD. a) Cell cycle distribution and b) apoptotic cells were detected through PI staining and Annexin V/PI, respectively. c) Related proteins were detected by Western blot. **p* < 0.05, ***p* < 0.01, ****p* < 0.001 *vs*. control group.

reduced compared with NCTD treated alone (p < 0.05), implying that NCTD-induced G2/M arrest could be partially abolished by SP600125. Annexin V/PI results showed that SP600125 could partially reverse NCTD-induced apoptosis (Fig. 5b). In addition, NCTD-induced alternations in the expression levels of cyclinB1, pCDC2, pCDC25C, active caspase-3/9 and cleaved PARP were restored by SP600125 pretreatment (Fig. 5c,d). Similarly, pretreatment of ACHN cells with SCH772984 also partially diminished the NCTD-induced increase of G2/M cells and apoptosis (Fig. 6). These results clearly implied that the activa-

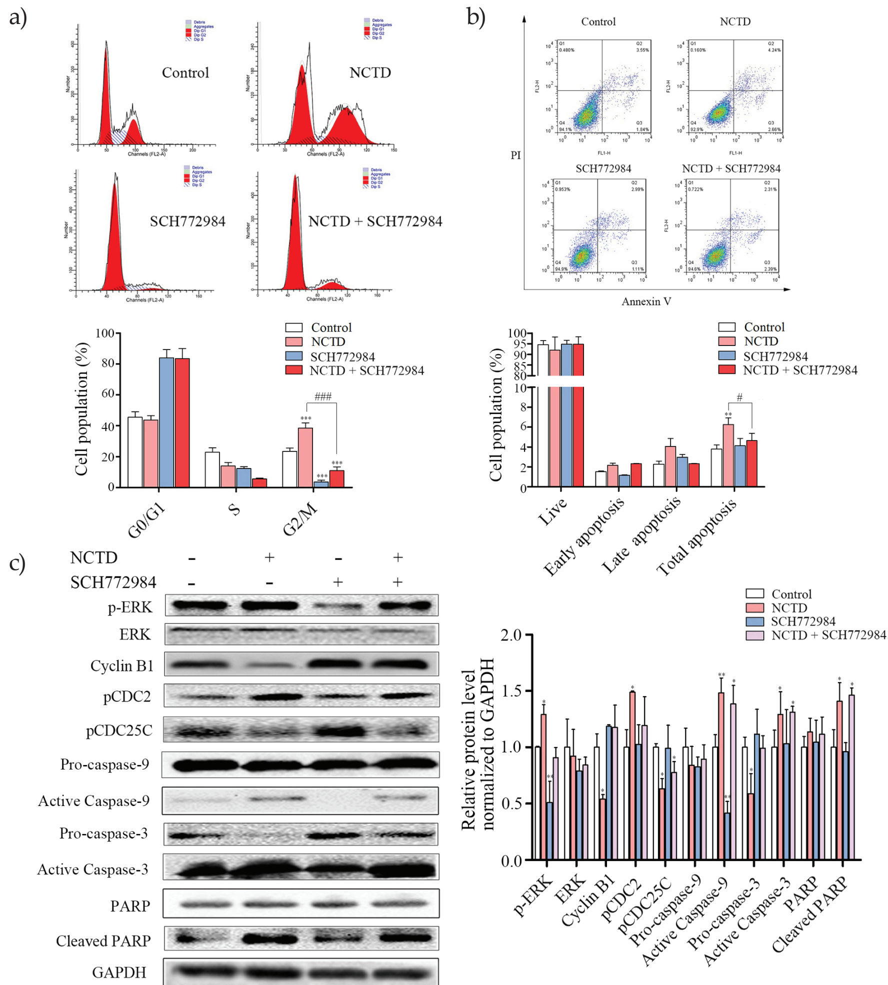

Fig. 6. SCH772984 attenuate NCTD-induced G2/M arrest and apoptosis. RCC cell lines were pretreated with 1 μmol L^{-1} SCH772984 before being exposed to NTCD. a) Cell cycle distribution and b) apoptotic cells were detected through PI staining and Annexin V/PI, respectively. c) Related proteins were detected by Western blot. *p < 0.05, **p < 0.01, ***p < 0.001 *vs*. control group 9.

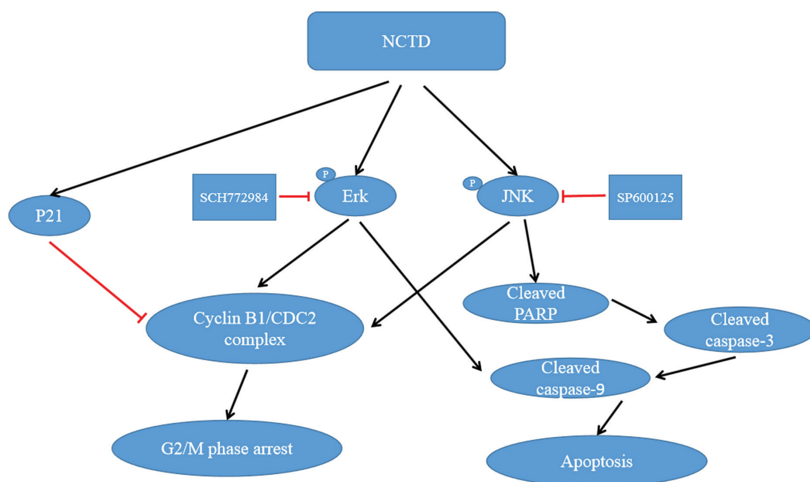

Fig. 7. Overview of pathways for NCTD-induced G2/M phase arrest and apoptosis in RCC cells.

tion of ERK and JNK signaling pathways were involved in NCTD-induced G2/M cell cycle arrest and apoptosis. Mechanistically, NCTD had induced RCC cells apoptosis and G2/M arrest through triggering modulations of proteins related to cell cycle and DNA damage (Fig. 7).

CONCLUSIONS

In summary, the present study shows that the mechanism of NCTD antitumor activity in ACHN cells is mediated by the ERK and JNK signaling pathways that are involved in G2/M cell cycle arrest and apoptosis. The results presented confirm the antitumor potential of NCTD for RCC.

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Supplementary material available upon request.

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