

Nitidine chloride inhibits proliferation, induces apoptosis via the Akt pathway and exhibits a synergistic effect with doxorubicin in ovarian cancer cells

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Abstract. Nitidine chloride (NC) exhibits anti-tumor properties in various types of tumor. However, to the best of our knowledge there is no previous evidence of NC involvement in the apoptosis or proliferation of ovarian cancer cells and the underlying molecular mechanisms. The present study aimed to investigate the influence of NC on the viability and apoptosis of ovarian cancer cells and the synergistic effect NC and doxorubicin (DOX) may have on ovarian cancer cells. The viability and proliferation of ovarian cancer cells were examined using a methyl thiazolyl tetrazolium assay and ³H-thymidine incorporation assay. The apoptotic rate of ovarian cancer cells was detected by flow cytometry. The expression of apoptosis-associated proteins and Akt serine/threonine kinase 1 (Akt) were determined by western blot analysis following NC treatment. The inhibitory effect of NC on the proliferation of ovarian cancer cells was demonstrated in a time and dose-dependent manner. The pro-apoptotic effect of NC on ovarian cancer cells was also observed. It was determined that NC significantly downregulated the protein expression levels of B-cell CLL/lymphoma 2 (Bcl-2) and upregulated the expression of Bcl-2-associated X protein, p53, caspase-3 and -9. NC suppressed Akt phosphorylation. Additionally, the present study demonstrated that the effect of NC on the proliferation and apoptosis of

ovarian cancer cells was Akt-dependent by using the phosphatidylinositol-4,5-bisphosphate 3-kinase/Akt signaling pathway inhibitor, LY294002. NC exhibited a synergistic inhibitory effect on the viability of ovarian cancer cells when combined with DOX. The current study demonstrated that NC inhibited the proliferation and induced the apoptosis of ovarian cancer cells via the Akt signaling pathway and highlighted its potential clinical application for the treatment of ovarian cancer.

Introduction

Ovarian cancer is a lethal gynecological malignancy and the fifth leading cause of cancer-associated mortality worldwide. In 2013, there were >2 million novel cases of ovarian cancer and 1 million mortalities worldwide (1-3). Chemotherapy following surgical resection is the current primary treatment for localized ovarian cancer. Progress has been made in early diagnosis of patients; however, treatment options for the majority of patients with advanced stage ovarian cancer remain limited. Particularly, resistance to chemotherapeutic drugs often leads to poor prognosis (4-6). Therefore, it is important to investigate the potential molecular mechanisms underlying the pathogenesis of ovarian cancer, and to identify novel therapeutic agents and combination regimens in order to improve the treatment and prognosis of patients with ovarian cancer.

Nitidine chloride (NC) (Fig. 1A) is isolated from the root of *Zanthoxylum nitidum* (Roxb). Numerous pharmacological properties of NC have been previously reported, including anti-oxidant, anti-inflammatory, anti-fungal, analgesic and anti-human immunodeficiency virus functions (7,8). Previous studies have determined that NC exhibits anti-tumor activity in several types of cancer. Additionally, NC inhibits cell proliferation and induces apoptosis in renal cancer, gastric cancer, breast cancer and hepatocellular carcinoma (9-12). NC was also capable of suppressing the invasion and metastasis of renal cancer via the Akt serine/threonine kinase 1 (Akt) pathway, and breast cancer by inhibiting the SRC proto-oncogene, non-receptor tyrosine kinase/focal adhesion

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kinase-associated signaling pathway (13,14). However, to the best of our knowledge, no evidence has previously reported whether NC exerts any effect on ovarian cancer proliferation, apoptosis and its underlying mechanisms. NC may be successful in treating ovarian cancer; however, the synergistic effect of NC and other therapeutic agents requires further investigation.

The present study investigated the effects of NC on ovarian cancer cell proliferation and apoptosis. It was also determined that the Akt pathway is important for NC-induced apoptosis. Additionally, the synergistic effect of NC with doxorubicin (DOX) on ovarian cancer cells was evaluated.

Materials and methods

Cell lines and reagents. The A2780 human ovarian cancer cell line was purchased from the American Type Culture Collection (Manassas, VA, USA) and cultured in RPMI-1640 (Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA) containing 10% fetal bovine serum (FBS; Invitrogen; Thermo Fisher Scientific, Inc.), 100 U/ml penicillin, and 100 µg/ml streptomycin in 5% CO₂ at 37°C. Rabbit anti-Akt (cat. no. 4685), phosphorylated-Akt (cat. no. 4058), B-cell CLL/lymphoma 2 (Bcl-2; cat. no. 2870), Bcl-2-associated X protein (Bax; cat. no. 502), p53 (cat. no. 2527), caspase-3 (cat. no. 9665) and -9 (cat. no. 9502) antibodies were purchased from Cell Signaling Technology, Inc. (Danvers, MA, USA). NC was purchased from Tauto Biotech Co., Ltd. (Shanghai, China) and dissolved in dimethyl sulfoxide (DMSO). LY294002, a selective phosphatidylinositol-4,5-bisphosphate 3-kinase (PI3K) Akt inhibitor, was purchased from Sigma-Aldrich (St. Louis, MO, USA). Annexin V-fluorescein isothiocyanate (FITC)/propidium iodide (PI) apoptosis detection kit was purchased from BD Biosciences (Franklin Lakes, NJ, USA).

Methyl thiazolyl tetrazolium (MTT) assay of cell viability and proliferation. Cell viability and proliferation were detected by using MTT assay. A2780 cells in 100 µl RPMI-1640 were seeded at a density of 5.0×10^3 cells/well in 96-well plates. Following treatment with NC (0, 2.5, 5, 10, 20 or 50 µM) and/or DOX (0, 0.25, 0.5, 1, 2, 5 µM; Aladdin Reagents Co., Ltd., Shanghai, China) for different time-points (24, 48 or 72 h), 20 µl MTT (5 mg/ml) was added to each well. Subsequently, the cells were incubated for 4 h, then 100 µl DMSO was added to each well for another 15 min. Finally, the absorbance values were detected using a microplate luminometer (Bio-Rad Laboratories, Inc., Hercules, CA, USA) at 490 nm.

³H-thymidine incorporation assay. A2780 cells were cultured in RPMI-1640 with 10% FBS at 50% confluence. Next, the A2780 cells were cultured in serum-free RPMI-1640 for 24 h and treated with NC at different concentrations (0, 2.5, 5, 10, 20 µM) for 40 h. ³H-thymidine (final concentration 1 µCi/ml) was added to the media during the last 24 h of culturing. The cells were then washed with ice-cold phosphate-buffered saline (PBS), and precipitated with ice-cold 5% trichloroacetic acid (TCA; Sigma-Aldrich) for 4 h. The cells were washed with ice-cold 5% TCA twice,

followed by washed with ice-cold PBS. Subsequently, the cells were lysed using 200 µl 0.5 M NaOH for 30 min at 37°C. DNA synthesis was determined by ³H-thymidine uptake using a liquid scintillation counter (LS-6500; Beckman Coulter, Inc., Brea, CA, USA).

Western blot analysis. Total cell protein concentrations were detected by using the Pierce bicinchoninic acid protein assay kit (Thermo Fisher Scientific, Inc.). Equal quantity of protein from cell lysates (10 µg) were loaded on 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis gels. Following electrophoresis, proteins were transferred to polyvinylidene fluoride membranes (EMD Millipore, Billerica, MA, USA), blocked with 5% fat-free milk at room temperature for 1 h, and incubated with the aforementioned primary antibodies overnight at 4°C. The membranes were subsequently washed with Tris-buffered saline Tween-20 and incubated with horseradish peroxidase-conjugated goat anti-rabbit secondary antibodies (cat. no. ZDR-5306; OriGene Technologies, Inc., Shanghai, China) for 1 h at room temperature. Immune complexes were detected with enhanced chemiluminescence reagents (EMD Millipore), and the blots were quantified by densitometric analysis using the AlphaImager IS 2200.

Apoptosis analysis by Annexin V/PI assay. Apoptosis of A2780 cells was detected by using the Annexin V-FITC/PI assay. A2780 cells were seeded into 6-well plates at a density of 1.0×10^6 cells/well. Following NC treatment (0, 10, 20 µM) for 24 h the A2780 cells were harvested, washed and resuspended in PBS. Apoptotic cells were determined using an Annexin V-FITC apoptosis detection kit according to the manufacturer's protocol. Briefly, the A2780 cells were washed and then incubated for 15 min at room temperature in the dark in 200 µl 1X binding buffer containing 5 µl Annexin V-FITC and 10 µl PI. The apoptotic rate was detected by the BD Accuri C6 flow cytometer (BD Biosciences) and processed using FlowJo software (version 7.0; Tree Star, Inc., Ashland, OR, USA).

Statistical analysis. Data are expressed as the mean ± standard deviation. All of the experiments were repeated at least three times. Comparisons among values for all groups were performed using a one-way analysis of variance with Dunnett's test for comparison of multiple groups. $P < 0.05$ was considered to indicate a statistically significant difference.

Results

NC inhibits the proliferation of ovarian cancer cells. An MTT assay was performed to determine the effect of NC on ovarian cancer cell proliferation. As presented in Fig. 1B, NC significantly reduced the proliferation of ovarian cancer cells in a time- and dose-dependent manner compared with the control group ($P < 0.01$). To verify the aforementioned findings, a ³H-thymidine incorporation assay was used to determine the importance of NC on the proliferation of ovarian cancer cells. As presented in Fig. 1C, NC (2.5, 5, 10 and 20 µM) significantly suppressed the proliferation of ovarian cancer cells compared with the control ($P = 0.038$, $P = 0.0071$, $P = 0.0062$, $P = 0.0050$, respectively). The 10 and 20 µM NC concentrations

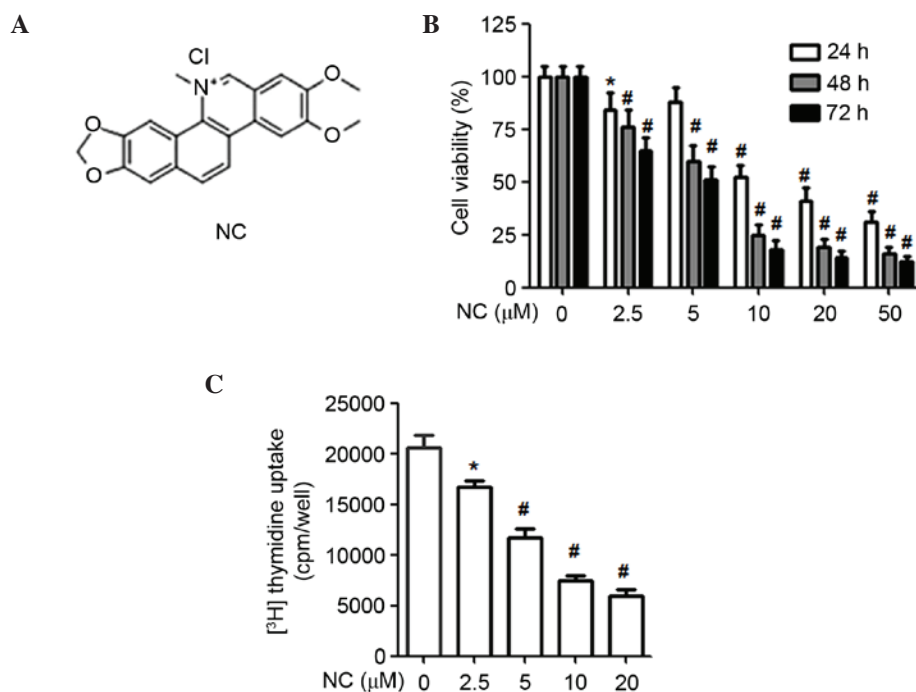


Figure 1. NC inhibited the proliferation of A2780 ovarian cancer cells. (A) Chemical structure of NC. (B) NC treatment for different time periods (24, 48 and 72 h) and different concentration gradients (0, 2.5, 5, 10, 20 and 50 μ M), methyl thiazolyl tetrazolium assay was used to determine the viability of A2780 cells. (C) Following 40 h treatment with NC at different concentrations (0, 2.5, 5, 10 and 20 μ M), 3 H-thymidine incorporation assay was used to detect the proliferation of A2780 cells. * $P < 0.05$, # $P < 0.01$ vs. control group. Data are presented as the mean \pm standard deviation of three independent experiments. NC, nitidine chloride.

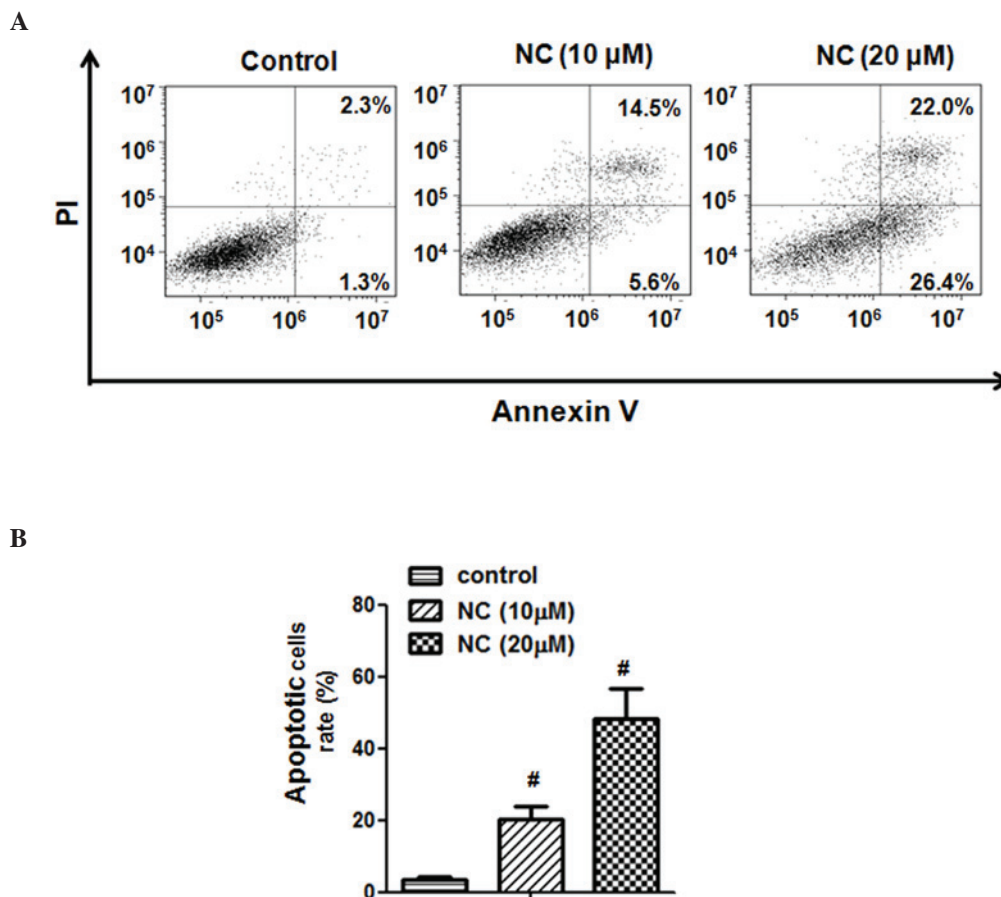


Figure 2. NC induced dose-dependent apoptosis in A2780 ovarian cancer cells. (A) A2780 cells were treated with NC (0, 10 and 20 μ M) for 24 h and double-stained with Annexin V and PI. Then the A2780 cells were analyzed with a flow cytometer. (B) The bar graph represents the mean \pm standard deviation of three independent experiments. # $P < 0.01$ vs. the control group. NC, nitidine chloride; PI, propidium iodide.

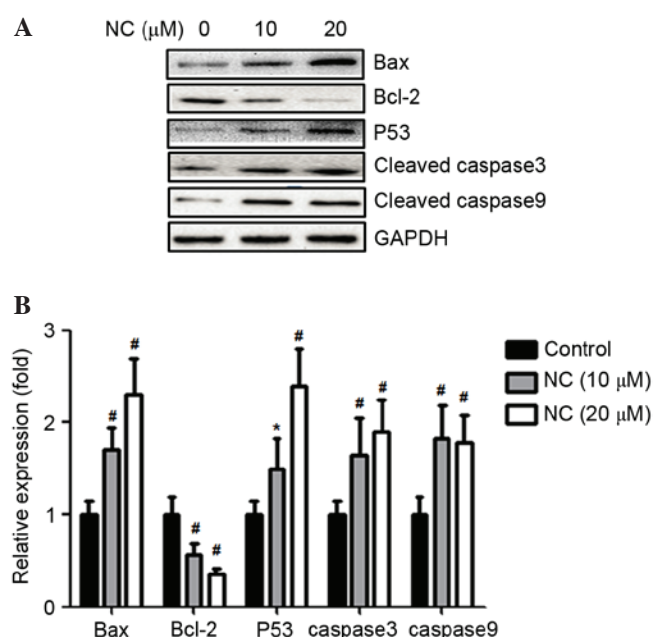


Figure 3. NC reduced the expression of Bcl-2 and increased the expression of Bax, Bcl-2, p53, caspase-3 and -9 in A2780 ovarian cancer cells. (A) A2780 cells were treated with NC (0, 10 and 20 μ M) for 24 h. The expression levels of Bax, Bcl-2, p53, cleaved caspase-3 and -9 in cell lysates were detected using western blot analysis. (B) Statistical analysis of the western blot results. *P<0.05, #P<0.01 vs. control group. Data are presented as the mean \pm standard deviation from three independent experiments. NC, nitidine chloride; Bax, Bcl-2-associated X protein; Bcl-2, B-cell CLL/lymphoma 2.

were selected for the subsequent experiments. These data suggested that NC effectively suppressed the proliferation of ovarian cancer cells.

NC induces apoptosis in ovarian cancer cells. To confirm that the inhibitory effect of NC on the proliferation of A2780 ovarian cancer cells was due to the direct effect on apoptosis, the A2780 cells were treated with NC (0, 10 and 20 μ M) for 24 h. As presented in Fig. 2 it was determined that 10 and 20 μ M NC significantly promoted the apoptosis of ovarian cancer cells compared with the control group (P=0.0035 and P=0.0010, respectively). The apoptotic rate of ovarian cancer cells increased from 2.3 ± 1.3 (control) to 14.5 ± 5.6 and $22.0 \pm 26.4\%$ following treatment with 10 and 20 μ M, respectively, for 24 h. These findings demonstrated the anti-tumor effect of NC on ovarian cancer cells.

NC induces the apoptosis of ovarian cancer cells via altered expression levels of apoptosis-associated proteins. In order to determine the underlying mechanism of ovarian cancer cell apoptosis induced by NC, the protein expression levels of Bax, Bcl-2, p53, caspase-3 and -9 were determined. Western blot analysis demonstrated that, following treatment with NC (10 and 20 μ M), the expression levels of pro-apoptotic protein Bax were significantly upregulated (P=0.0074 and P=0.0058, respectively; Fig. 3) and the expression levels of the anti-apoptotic protein, Bcl-2 were significantly downregulated compared with the control group (P=0.0062 and P=0.0044, respectively; Fig. 3). Simultaneously, the protein expression levels of p53 (10 μ M NC, P=0.031; 20 μ M NC, P=0.0040), caspase-3 (10 μ M NC, P=0.0054; 20 μ M NC, P=0.0057)

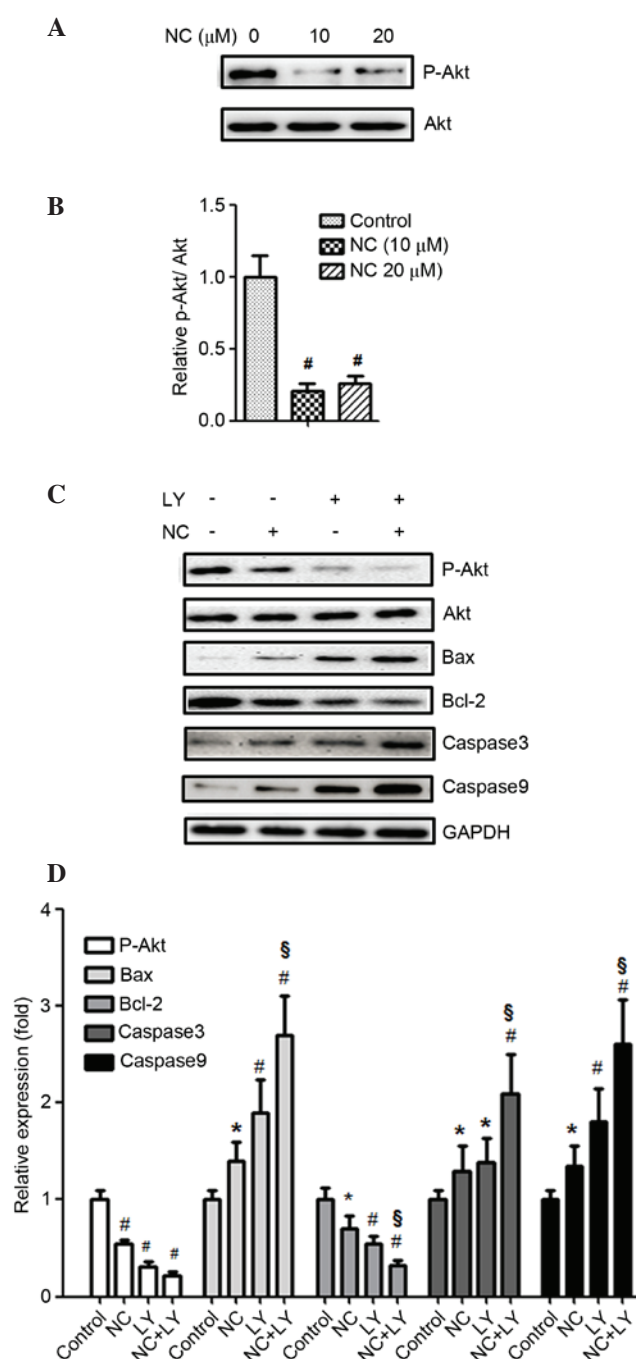


Figure 4. NC inhibited Akt phosphorylation and the pro-apoptotic effect on A2780 cells was Akt-dependent. (A) A2780 cells were treated with NC (0, 10 and 20 μ M) for 24 h and the levels of p-Akt, Akt were analyzed by western blotting. (B) Statistical analysis of the western blotting results. #P<0.01 vs. control group. (C) A2780 cells were pretreated with the Akt inhibitor LY (50 μ M) for 30 min followed by incubation with or without NC (20 μ M) for 24 h. The expression levels of p-Akt, Akt, Bax, Bcl-2, caspase-3 and -9 were detected by western blotting with GAPDH as an internal control. (D) Statistical analysis of the western blotting results. *P<0.05, #P<0.01 vs. control group, §P<0.01 vs. NC-treated group. Data are presented as the mean \pm standard deviation from three independent experiments. p-Akt, phosphorylated-AKT serine/threonine kinase 1; NC, nitidine chloride; Bax, Bcl-2-associated X protein; Bcl-2, B-cell CLL/lymphoma 2; LY, LY294002.

and -9 (10 μ M NC, P=0.0055; 20 μ M NC, P=0.0054) were also significantly upregulated compared with the control group (Fig. 3).

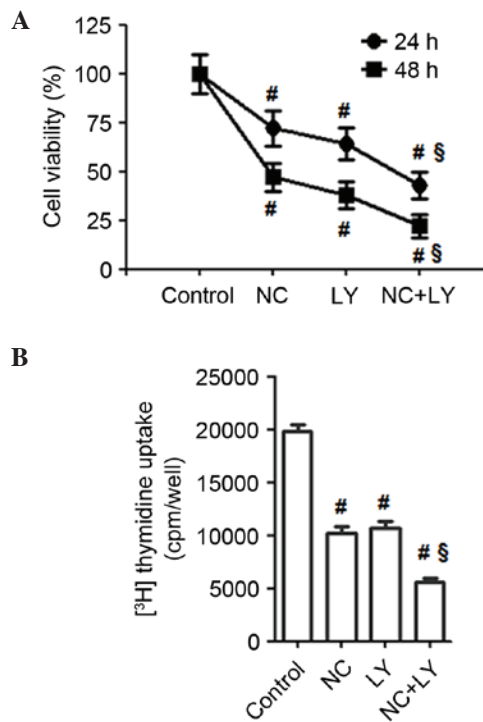


Figure 5. NC inhibited the proliferation of A2780 cells. (A) A2780 cells were pretreated with LY (50 μ M) for 30 min followed by incubation with or without NC (20 μ M) for 24 and 48 h, cell viability was determined by methyl thiazolyl tetrazolium assay. (B) A2780 cells were pretreated by using LY (50 μ M) for 30 min followed by incubation with or without NC (20 μ M) for 40 h, then cell proliferation was determined by 3 H-thymidine incorporation assay. Data are presented as the mean \pm standard deviation from three independent experiments. [#]P<0.01 vs. control group, ^{\$}P<0.01 vs. NC-treated group. NC, nitidine chloride; LY, LY294002.

NC inhibits Akt phosphorylation in ovarian cancer cells. The Akt signaling pathway is important for tumor progression. To determine the molecular mechanism involved in the effect of NC on the proliferation and apoptosis of ovarian cancer cells, the Akt signaling pathway was examined. As presented in Fig. 4A and B, Akt phosphorylation was significantly downregulated by treatment with NC (10 and 20 μ M; P=0.0041 and P=0.0045, respectively) for 24 h, compared with the control group (P<0.01). This indicated that the Akt signaling pathway may be involved in the viability and apoptosis of ovarian cancer cells.

Suppression of the Akt signaling pathway enhanced the pro-apoptotic and anti-proliferative effect of NC on ovarian cancer cells. LY294002, a PI3K inhibitor, was used to stop Akt phosphorylation. As presented in Fig. 4C and D, the inhibition of Akt phosphorylation with LY294002 significantly enhanced the NC-induced downregulation of the Bcl-2 expression levels compared with NC treatment only (P=0.0061), whereas the expression levels of Bax, caspase-3 and -9 were significantly upregulated by LY294002 + NC treatment compared with 20 μ M NC treatment alone (P=0.0033, P=0.0041 and P=0.0052). These findings indicated that NC-induced apoptosis may be PI3K/Akt-dependent. Furthermore, the effect of NC on the proliferation of ovarian cancer cells and the PI3K/Akt signaling pathway was determined using an MTT assay (Fig. 5A) and 3 H-thymidine incorporation assay (Fig. 5B). As presented in Fig. 5A, the

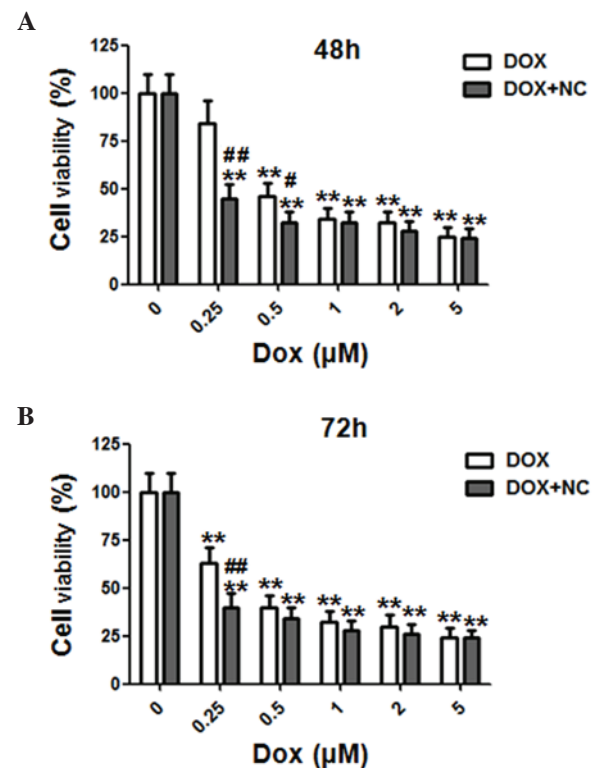


Figure 6. Synergistic effect of NC and DOX on the viability of A2780 cells. Subsequent to treatment with DOX alone (0.25, 0.5, 1, 2, 5 μ M) or in combination with NC (2 μ M) for (A) 48 h and (B) 72 h, A2780 cell proliferation was detected using an methyl thiazolyl tetrazolium assay. Data are presented as the mean \pm standard deviation of three independent experiments. ^{**}P<0.01 vs. 0 μ M controls; [#]P<0.05, ^{##}P<0.01 vs. DOX-treated group. DOX, doxorubicin; NC, nitidine chloride.

PI3K/Akt inhibitor LY294002, significantly enhanced the NC-induced suppression of the proliferation of ovarian cancer cells compared with the control and the NC-only treated group (P=0.0057, 24 h; P=0.0051, 48 h). As demonstrated in Fig. 5B, LY294002 enhanced 3 H-thymidine incorporation compared with NC alone (P=0.0066). Therefore, blocking Akt activation may significantly enhance the NC-induced suppression of the proliferation of ovarian cancer cells.

NC exhibits a synergistic effect with DOX on limiting cell viability of ovarian cancer cells. A previous study on anti-tumor agents focused on limiting drug resistance of traditional chemotherapeutic agents by combining with novel anti-tumor drugs to achieve optimal curative effects (14). In order to investigate the synergistic effect of NC and DOX on A2780 ovarian cancer cells, the cells were treated with various doses of DOX (0, 0.25, 0.5, 1, 2, 5 μ M) and NC (2 μ M) for 48 and 72 h (Fig. 6A and B). The cell viability was examined by MTT assays. DOX alone or in combination with NC (2 μ M) inhibited the viability of ovarian cancer cells in a time- and dose-dependent manner. Notably, the viability of A2780 cells was decreased from 84.2 \pm 11.2 to 43.5 \pm 7.1 (P=0.0063), and 45.7 \pm 6.8 to 31.8 \pm 5.3% (P=0.034), respectively, when treated with 0.25 and 0.5 μ M DOX, in combination with NC (2 μ M) at 48 h. For the 72 h treatment, the viability of A2780 cells decreased from 55.9 \pm 9.2 to 44.6 \pm 6.1% (P=0.0083) when treated with 0.25 μ M DOX in combination with NC (2 μ M).

These findings indicated that the combination of DOX and NC exerted a synergistic inhibitory effect on the viability of the ovarian cancer cells.

Discussion

Uncontrolled proliferation and resistance to apoptosis of tumor cells is considered to contribute to mortality in patients with ovarian cancer (15,16). Conventional chemotherapeutic agents have limited long-term application due to their side effects. Therefore, understanding the molecular mechanisms of proliferation and apoptosis, and developing effective approaches to inhibit proliferation and induce apoptosis of cancer cells are the crucial for cancer research. Therefore, investigations of the therapeutic features of natural products in tumor therapy are frequently performed (17,18). As a bioactive phytochemical alkaloid extracted from *Zanthoxylum nitidum* (Roxb), NC exhibits multiple pharmacological effects on oxidation and inflammation (7,8). Previous studies have demonstrated the capacity of NC to induce apoptosis and inhibit of proliferation, migration and invasion in breast, renal, hepatocellular carcinoma and gastric cancer cells (9-14). However, the effect of NC on ovarian cancer apoptosis and proliferation had not been fully elucidated and the molecular mechanisms were unclear. To the best of our knowledge, the current study was the first to demonstrate that NC may effectively suppress the viability of ovarian cancer cells and induce their apoptosis. The mechanism of proliferation inhibition by NC was due to downregulation of the Akt signaling pathway. Therefore, a novel molecular mechanism that allows NC to exhibit the anti-tumor activity in ovarian cancer cells was identified.

Cell apoptosis is regulated by various factors, including Bcl-2 protein family members and caspases (19). The Bcl-2 protein family members, including the anti-apoptotic proteins (Bcl-2, Bcl-2-like 1, Bcl-2-like 2 and myeloid cell leukemia 1) and pro-apoptotic proteins [Bax and Bcl-2-associated agonist of cell death (Bad)] are important for apoptosis and act via caspases (19,20). Previous studies have determined that in the process of apoptosis, the release of cytochrome C was regulated by Bax mitochondria translocation and oligomerization, which in turn activated caspase-3 and -9 (21,22). Subsequently, cell apoptosis is triggered by the inhibition of Bcl-2 and Bax oligomerization. Thus, Bcl-2 was understood to stabilize the mitochondrial membrane by blocking internal calcium release into the cytoplasm (23,24). A previous study demonstrated that the tumor suppressor, p53, was associated with several Bcl-2 protein family members involved in cell apoptosis (25). Previous studies have determined that NC inhibited the proliferation of renal and breast cancer cells by inducing apoptosis, and regulating the expression levels of Bcl-2 and Bax (9,12). The present study demonstrated that NC inhibited the proliferation of ovarian cancer cells and induced their apoptosis in a time- and dose-dependent manner. The expression levels of apoptosis-associated proteins (Bax and Bcl-2) in ovarian cancer cells, treated with NC were also investigated. It was determined that NC altered the expression level ratio of Bax to Bcl-2. Additionally, caspase-3 and -9 were activated by NC treatment, which resulted in the apoptosis of ovarian cancer cells. The current study also examined the effect of NC on the expression levels of p53 in ovarian cancer cells and

demonstrated the upregulation of p53 in a dose-dependent manner. These results indicated that NC-induced apoptosis of ovarian cancer cells may be a result of the imbalance between Bcl-2 and Bax expression levels, and the activation of caspase-3 and -9. Additionally, p53 was also demonstrated to be involved in NC-induced apoptosis of ovarian cancer cells.

Akt has been previously established as an important regulator of cell proliferation and survival (26). A previous study demonstrated that Akt mediated cell survival and apoptosis via its downstream targets, including Bad and caspase-9 (27). Another previous study indicated that Akt affected the regulation of Bax activity (28). The present study determined that the expression of p-Akt was decreased when cells were treated with NC. To determine whether the Akt signaling pathway was involved in NC-induced apoptosis of ovarian cancer cells and inhibited their proliferation, Akt phosphorylation was blocked by using the PI3K/Akt inhibitor, LY294002. It was demonstrated that the inhibition of Akt activity by LY294002 enhanced the apoptotic rate induced by NC, which was determined by western blot analysis and MTT assays. These results suggested that NC-induced apoptosis of ovarian cancer cells by inhibition of Akt phosphorylation and altering the expression levels of the Bcl-2 protein family members. The PI3K/Akt inhibitor (LY294002) enhancing the apoptotic rate induced by NC also suggested that Akt was located upstream of Bcl-2 and Bax.

To reduce the resistance to therapeutic agents, combination therapy is frequently used in cancer treatment (29). The anticancer agent DOX is highly effective for the treatment multiple types of cancer. However, there are problems with side effects, including acute and chronic cardiotoxicity, and neutropenia, which significantly limit its chemotherapeutic usage (30). Thus, it is important to develop combination therapies in order to decrease the side effects of chemotherapy agents. The present study determined the synergistic effect of NC and DOX in ovarian cancer cells. NC exerted a synergistic inhibitory effect on the viability of ovarian cancer cells and enhanced the anti-tumor effect when administered in conjunction with a low dose of DOX. This indicates that a combination of NC and DOX at certain concentrations may reduce its side effects. The precise mechanisms by which NC improves chemotherapeutic efficacy require further investigation.

In conclusion, to the best of our knowledge, the present study was the first to demonstrate that NC suppressed the proliferation of ovarian cancer cells and induced their apoptosis. Additionally, the effect of NC was demonstrated to be mediated by the Akt signaling pathway. NC exhibited a synergistic effect with DOX in ovarian cancer cells. Therefore, it was determined that NC is a promising agent for ovarian cancer therapy and additional *in vivo* studies are required to confirm its efficacy.

Acknowledgements

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