Inhibitory Effect of the Zinc Metallochaperone NSC319726 on Ovarian Cancer Cells via the Regulation of P53

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Abstract

Ovarian cancer is the leading cause of death from malignancies of the female reproductive system. In recent years, there has been little development regarding the treatment of ovarian cancer. Wild-type tumor protein p53 (P53) can inhibit the development of tumor, however, mutations in P53 have been shown in most cases of ovarian cancer. The mutated gene encoded P53 transforms from a tumor suppressor gene to an oncogene, losing its original anti-tumor function. Studies have shown that the zinc metallochaperone NSC319726 can promote the correct folding of P53 in cancer cells and restore its physiological function, however, the function of NSC319726 in ovarian cancer has not been elaborated. So we investigated the role of NSC319726 on biological functions of ovarian cancer and preliminarily determined the specific molecular mechanism. The results showed that NSC319726 could inhibit proliferation, migration and invasion of ovarian cancer cells and promote their apoptosis. Mechanically, NSC319726 regains the tumor-suppressed function of P53, further activates the downstream cyclin-dependent kinase CDK inhibited protein P21, thereby blocking the cell cycle and inhibiting cells proliferation. Therefore, NSC319726 has the potential to act as a novel drug for treating ovarian cancer.

Introduction

Ovarian cancer (OC) is the leading cause of death from malignancies of the female reproductive system, with a 5-year survival rate of approximately 47% [1]. According to statistics, there are about 2.39 million new cases and 1.52 million deaths annually worldwide [2], with epithelial ovarian cancer having the highest mortality rate among all types of gynecologic tumors. Since the ovary is located in the deep part of the pelvic cavity, symptoms appear late when lesions occur, and most ovarian patients are diagnosed at an advanced stage, when the metastases of tumor are already present in OC patients [3–5]. The traditional treatments to ovarian cancer are mainly surgery and adjuvant chemotherapy, and most patients can get good treatment effects when they first receive platinum-based chemotherapy, but as the number of chemotherapy increases, the development of chemotherapy resistance may reduce the prognosis of patients [6–8]. Therefore, there is an urgent need to explore novel and effective drugs to improve treatments to ovarian cancer.

Wild-type P53 is involved in cell cycle regulation, DNA repair, apoptosis and gene transcription for senescence [9]. Some studies have shown that mutations in P53 occur in most cases of ovarian cancer [10]. Mutated P53 loses its original biological function and can show functional changes such as loss-of-function (LOF), gain-of-function (GOF) or dominant negative effect (DNE). These changes may promote tumor development to some extent [11]. NSC319726, a zinc metallochaperone whose molecular structure is shown in the figure (Fig. 1a), can restore P53 with missense mutations to wild-type P53 by chelating zinc ions at an optimal concentration to promote proper P53 folding [12], which further suppresses the proliferation of tumor cells and promotes their apoptosis [13–14].

In this study, we first examined the effects of NSC319726 on the biological functions of ovarian cancer cells. The results showed that the proliferation, migration, invasion and apoptosis functions of ovarian

cancer cells were significantly altered under the effect of NSC319726. In addition, the anti-tumor function of P53 in ovarian cancer cells under NSC319726 treatment was also explored and verified by investigating the expression of cell cycle-related proteins which are regulated by P53. In conclusion, NSC319726 may have the possibility of becoming a novel therapeutic agent for ovarian cancer.

Materials And Methods

Cell culture

Human ovarian cancer cell lines SK-OV-3 and A2780 were purchased from Shanghai Biological Technology Co., Ltd. enzyme research (Shanghai, China). A2780 cells were maintained in RPMI 1640 medium (Corning, China) containing 10% fetal bovine serum (FBS, Biological Industries, Israel); SK-OV-3 were maintained in McCOY's 5A medium (JiNuo Biology, China) and IOSE80 were maintained in DMEM medium (Corning, China) containing 10% fetal bovine serum (FBS, Biological Industries, Israel). Cells were cultured in an incubator with 5% CO_2 at 37°C.

Reagents

NSC319726 was purchased from TargetMOI with an IC50 of 8 nM. Therefore, the concentration was set near the drug IC50 with isocratic settings of 5 nM, 10 nM, 20 nM, and 40 nM. After being diluted in DMSO solution, the drug was stored in a refrigerator at -20℃. The primary antibodies (GAPDH, P53, P21, P27, CDK2, CDK4, cyclinD1, cyclinE1) were bought from Proteintech, Wuhan, China, and stored in a refrigerator at -20℃.

Cell proliferation assay

A2780 and SK-OV-3 cells were introduced into a 96-well plate(5×10^3 cells/well)with medium of 100 μL per well. After the cells were completely attached, the medium of the experimental group was replaced with the new medium containing the corresponding concentration of the NSC319726. In the meantime, the control group was replaced with the medium added with DMSO solution of the same volume as the drug, and incubated for 24 hours. Then 10 μL of CCK assay solution was added to each well, mixed, and incubated for 2 hours in the dark, and cell OD values were measured using an enzyme marker at an absorbance of 450 nm. Cell viability % = [OD value of cells in the experimental group/mean OD value of cells in the control group] × 100.

Colony forming assay

A2780 and SK-OV-3 cells were introduced into a 6-well plate (1×10^3 cells per well). After one week, the medium of the experimental group was replaced with the new medium containing the corresponding concentration of the NSC319726, while the control group was replaced with the medium added with DMSO solution of the same volume as the drug, and the incubation was continued for 48 hours. After being washed with PBS for 3 times, the colonies were fixed in paraformaldehyde at room temperature for 15 minutes and then stained at room temperature for 30 minutes with 0.2% crystalline violet solution. The

6-well plates were gently rinsed with water until an excess crystalline violet solution was rinsed off and then were air-dried at room temperature. The plate was photographed under an Olympus CKX41 microscope and then measured using Image J software.

Wound healing assay

A2780 and SK-OV-3 cells were seeded into 6-well plates at 37°C under 5% CO2 for 24 hours. After that, the cells were scratched with a sterile micropipette tip and then washed with PBS, the experimental group was replaced with a new medium containing the corresponding concentration of the NSC319726, while the control group was replaced with the medium added with DMSO solution of the same volume as the drug. The cell migration distance was photographed by Olympus CKX41 microscope at 0, 12, 24 hours respectively, and then measured with Image J software (National Institute for Health, Bethesda, MD, USA). Migration rate (MR) was calculated by the formula of MR (%)=[(A-B)/A]×100(A is the width of 0 hours and B is the width of 24 hours).

Migration and invasion assays

Cell migration and invasion were evaluated using transwell chambers with 8 μm pores. 8×10^4 cells/well in serum-free medium were seeded into the upper chamber uncoated or coated with Matrigel (BD Biosciences, San Jose, CA, USA). NSC319726 with the corresponding concentration was added to the upper chamber of the experimental group, and an equal amount of DMSO solution was added to the upper chamber of the control group. The medium containing 20% fetal bovine serum was added to the lower chamber at 600 μL/well, then NSC319726 with the indicated concentrations was added to the lower chamber of the experimental group, and DMSO solution with equal concentrations was added to the lower chamber of the control group. After 24 hours of incubation, excess cells in the upper chamber were wiped off with a cotton swab, and the cells at the bottom of the small chamber were fixed with paraformaldehyde and stained with 0.2% crystal violet solution. The cell number was counted using a Zeiss Axioskop 2 plus microscope (Carl Zeiss, Thornwood, NY, USA).

Apoptosis assay

A2780 and SK-OV-3 cells were introduced into a 96-well plate at 1.5×10^4 cells/well. After the cells were fully attached, the medium of the experimental group was replaced with a new medium containing the corresponding concentration of the NSC319726, while the control group was replaced with medium added with DMSO solution of the same volume as the drug and incubated for 24 hours. After being washed by PBS, cells were fixed in 4% paraformaldehyde at room temperature for 30 minutes and then treated with 1% Triton X-100 solution at room temperature for 10 minutes. 50 μL TUNEL solution was added into each well, and the cells were incubated at 37°C for 60 minutes in the dark. Then the cell nuclei were stained with 4', 6-diamidino-2-phenylindole (DAPI) at room temperature for 5 minutes in the dark. Cells were observed under a fluorescent microscope (Eclipse Ti, Nikon, Melville, NY, USA). The percentage of TUNEL-positive cells was calculated using Image J software.

Immunoblotting analysis

Cells were collected and added with an appropriate amount of IP lysate (Beyotime, Shanghai, China) for total protein. Equal mass of protein in each sample was separated on SDS-PAGE and transferred to PVDF membrane (Millipore, Merck KgaA, Darmstadt, Germany). Then, the membrane was blocked in 5% skim milk at room temperature for 1 hour, after that, the membrane was incubated with primary antibodies (GAPDH, P53, P21, P27, CDK2, CDK4, cyclinD1, cyclinE1) at 1:1000 overnight at 4℃. Next, the membrane was incubated with HRP-conjugated Goat anti-rabbit IgG (H + L) secondary antibody (1:5000, Proteintech, Wuhan, China) at room temperature for 60 minutes followed by the detection of protein band after addition of ECL chemiluminescence (Meilunbio, Dalian, China).

Statistical analysis

All values were expressed as mean ± standard deviation (SD), and t-test was used for statistical comparison. An asterisk indicates statistical significance (*, $p < 0.05$; **, $p < 0.01$).

Results

NSC319726 suppresses a variety of ovarian cancer cells

To verify whether mutations in the tumor suppressor gene P53 are prevalent in ovarian cancer cells, we collected and analyzed the transcriptome data from The Cancer Genome Atlas (TCGA). 57 of the 65 ovarian cancer samples provided by TCGA held P53 mutations, accounting for about 87.7% of the total number of samples (Fig. 1b). Meanwhile, most of these mutations were missense mutations, which affected the biological function expression of P53 (Supplementary file 1). To detect the inhibitory effect of zinc ion chaperone NSC319726 on ovarian cancer cells, we collected and analyzed related data in Genomics of Drug Sensitivity in Cancer (GDSC), and found that most types of ovarian cancer cells were inhibited when treated with different concentrations of NSC319726 (Fig. 1c, Supplementary file 2).

NSC319726 suppresses ovarian cancer cells growth

To detect the effect of the NSC319726 on the proliferation of ovarian cancer cells A2780 and SK-OV-3, we performed a CCK-8 (Cell Counting Kit-8) assay. The results showed that, compared with the control group, the cell viability of cells in the experimental groups was significantly decreased under the effect of the drug, and the cell viability tended to decrease with the increase of drug concentration (Fig. 2a). To further visualize the effect of NSC319726 on the proliferation of ovarian cancer cells, we also performed a plate cloning assay to verify the suppressive effect of NSC319726 on ovarian cancer cells proliferation. The results exhibited the same trend as CCK-8 assays (Fig. 2b-c).

NSC319726 suppresses ovarian cancer cells migration and invasion

In order to detect the effect of NSC319726 on other biological functions of ovarian cancer cells, we investigated the effect of NSC319726 on migration and invasion of ovarian cancer cells. The results of Wound healing showed that, compared to the control group, the lateral migration rate of both A2780 and

SK-OV-3 cells in the experimental group was reduced under the effect of different concentrations of drugs (Fig. 3a-b). The same result was also presented in transwell assays (Fig. 3c-f). This indicates that the NSC319726 suppresses the migration and invasion of ovarian cancer cells A2780 and SK-OV-3.

NSC319726 promotes ovarian cancer cells apoptosis

TUNEL assay was performed to detect the effect of NSC319726 on apoptosis of ovarian cancer cells. The results showed that, compared with the control group, the percentage of apoptotic cells in A2780 and SK-OV-3 cells in the experimental group increased significantly under the effect of the drug, and the percentage of apoptotic cells gradually increased with the increase of drug concentration (Fig. 4a-b), indicating that the NSC319726 promotes apoptosis in ovarian cancer cells.

NSC319726 may suppress ovarian cancer cell proliferation through P53-regulated cell cycle arrest

P53 can further affect cell proliferation through the regulation of cell cycle proteins. To clarify the potential mechanism of the effect of NSC319726 on ovarian cancer cell proliferation, we performed protein immunoblotting. The results showed that, compared to the control group, the expression of P53 was increased in A2780 cells treated with NSC319726. Since CDK and cyclin were jointly involved in cell cycle regulation [15–16], the expression of P21, P27, cyclinE1, cyclinD1, CDK2 and CDK4 were detected, and the results showed that the expression of P21, P27 were increased, while cyclinE1, cyclinD1, CDK2 and CDK4 were reduced after drug treatment (Fig. 5a-b).

Discussion

Ovarian cancer is the leading cause of death among all types of female reproductive system tumors. In recent years, there has been little progress in the treatment of ovarian cancer [17–18]. Chemotherapy resistance is the biggest obstacle to the treatment of ovarian cancer. Despite an initial response rate of more than 80% to first-line chemotherapy, most patients eventually develop drug resistance and undergo cancer recurrence, so new treatments are urgently needed to improve survival in ovarian cancer [19]. In this study, we analyzed the effect of the zinc ion chaperone NSC319726 on ovarian cancer cells function and the possible mechanisms for its proliferation suppression.

P53 is a tetrameric multi-structured domain transcription factor, which plays an important role in maintaining cellular genomic integrity, and activates the expression of multiple genes. As a tumor suppressor gene, P53 encodes a DNA-binding transcription factor that induces cell growth arrest, cellular senescence, and cell death in response to cellular stress [20]. This is achieved through transcriptional regulation of multiple target genes, including P21, which blocks cell cycle progression, and Bax and Puma, which promote apoptosis [21]. This illustrates the critical role of P53 as a trigger that promotes cell cycle arrest or apoptosis during the development of cancer [22]. The mutational spectrum of P53 is atypical because tumorigenic alterations are overwhelmingly missense and map to nearly every position

within one of the domains of the protein (the DNA-binding domain). The DNA-binding domain possesses two unusual properties-one of the highest zinc affinities of any eukaryotic protein and extreme instability in the absence of zinc-which are predicted to poise P53 on the cusp of folding/unfolding in the cell, with a major determinant being available zinc concentration [23].

P53 gene is mutated in various tumors, such as serous endometrial cancer and ovarian cancer [24]. In almost all human cancers, P53 is inactivated, and in approximately 50% of human cancers, this inactivation is a direct result of mutations in the P53 gene [25–27]. The zinc ion (coordinated by amino acids C176 and H179 on the L2 loop and C238 and C242 on the L3 loop) plays a pivotal role in the tertiary structure of wild-type P53 and the correct folding of P53 [28–29]. Deficient zinc ion and excess zinc ion both result in P53 misfolding and loss of function, while the correct zinc ion concentration will reactivate P53 and inhibit tumor cell growth [30–31]. NSC319726, as a zinc metallochaperone, can provide the zinc ion of appropriate concentration and promotes correct folding of P53 by chelating zinc ion, thereby contributing to proper folding of P53 and restoration of its physiological function [29].

Results in this study demonstrated that NSC319726 can effectively suppress the proliferation, migration, invasion and promote apoptosis of ovarian cancer cells A2780 and SK-OV-3, and the suppressive effect becomes more pronounced as the drug concentration increases. The result of functional restoration of P53 is either tumor cell cycle arrest or suppression of tumor cell apoptosis [32]. It is thus seen that as the expression of P53 increases, the expression of its downstream CDK inhibitor protein P21 is overexpressed, which further inhibits the joint effort of CDK and cyclin. P21 and P27 inhibit the cyclin-CDK complex, causing retinoblastoma (Rb) to fail to fully phosphorylate, leading to G1/S phase block and causing cancer cells to undergo cycle arrest and fail to enter mitotic phase, thereby inhibiting their proliferation [32–36]. To explore the possible anti-proliferation mechanism of NSC319726 in ovarian cancer cells, we examined the effect of NSC319726 on the expression of P21, P27, CDK2, CDK4, cyclinD1, and cyclinE1. The overexpression of P21 and P27, and the down-regulation of CDK2, CDK4, cyclinD1, and cyclinE1 indicated that, NSC319726 can restore the function of P53, activate the CDK inhibitors P21 and P27, and inhibit CDK and cyclin, further suppress ovarian cancer cells growth. In addition, the activation of wild-type P53 may cause cell apoptosis through induction of a range of target genes such as BAX, PMAIP1 (NOXA), BBC3 (PUMA), P53AIP1, FAS, FDXR and TP53I3 (PIG3) [37], and the expression of wild-type P53 expression can promote MDM2-mediated Slug degradation to enhance E calmodulin expression thereby suppressing cell migration and invasion [38]. This is consistent with our observations that NSC319726 suppressed ovarian cancer cells migration and invasion, and promote their apoptosis. The exact mechanism needs to be further explored.

In summary, the zinc metallochaperone NSC319726 can suppress ovarian cancer cells proliferation, migration and invasion, and promote their apoptosis, and has the potential to be a novel drug for ovarian cancer treatment.

Declarations

Conflict of interest No potential conflicts of interest were disclosed.

Data availability All data generated or analyzed during this study are included in this published article.

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Figures

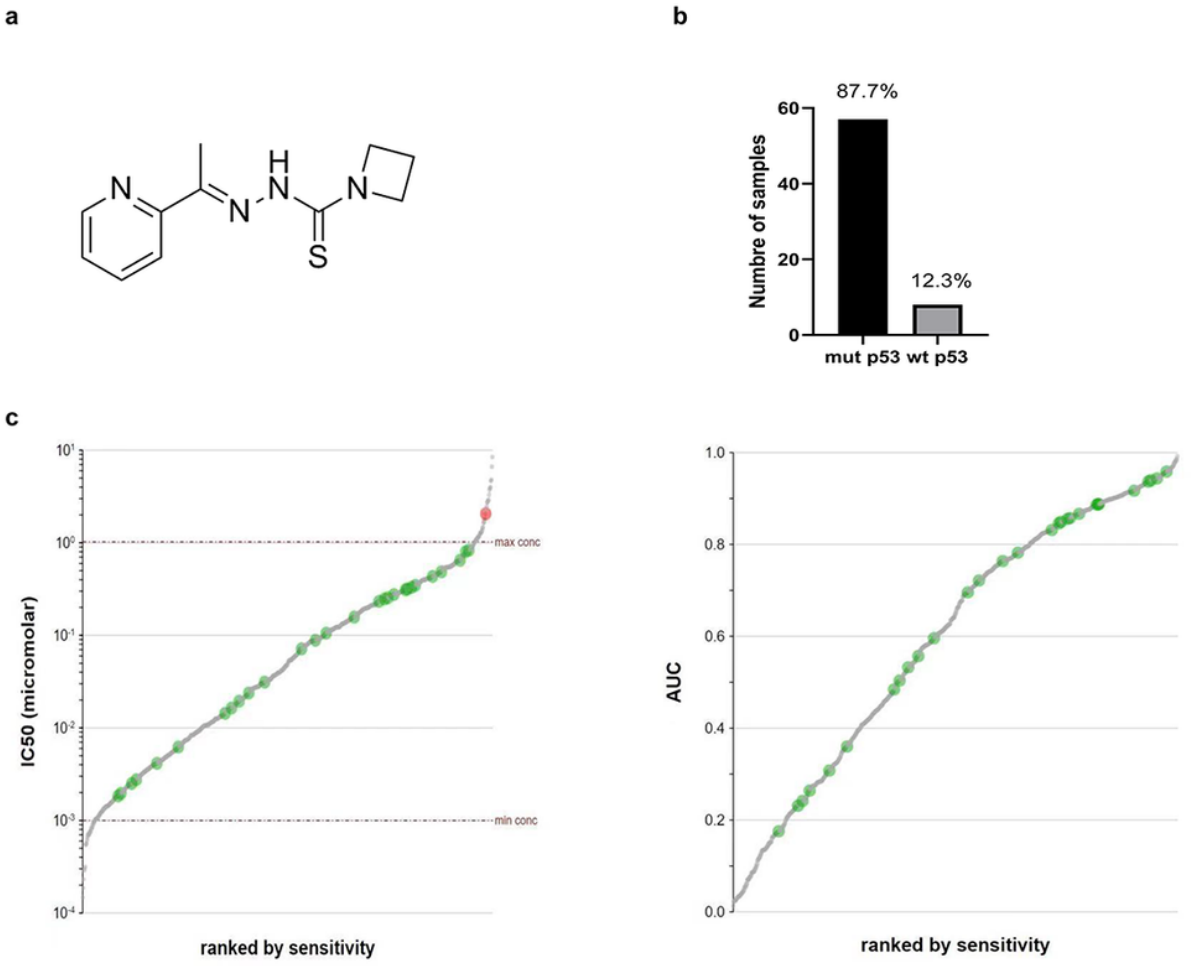

Figure 1

NSC319726 suppresses a variety of ovarian cancer cells. a Structure of NSC319726. b Analysis of the ratio of P53 mutations occurring in ovarian cancer cells according to TCGA data. c The IC50 and AUC of NSC319726 in treating different types of ovarian cancer cells according to GDSC data.

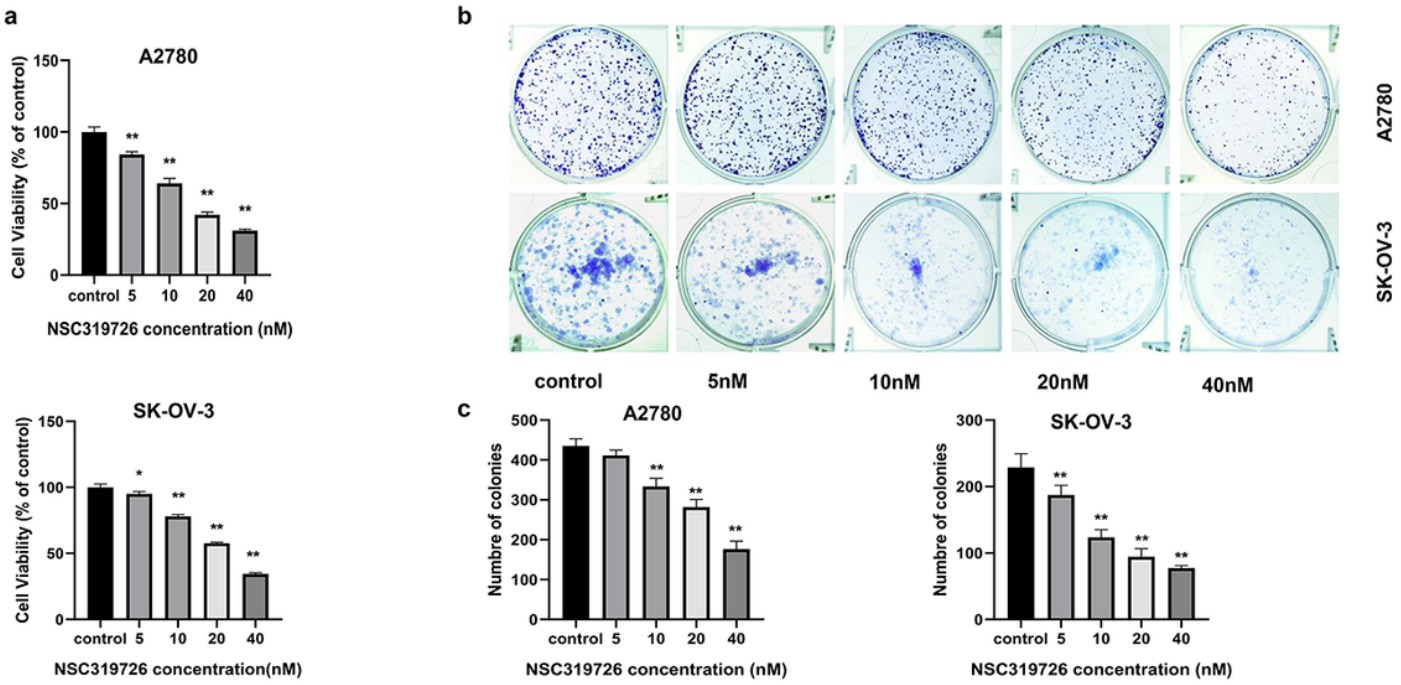

Figure 2

NSC319726 suppresses ovarian cancer cells growth. a The determination of the effect of different concentrations of drugs on the proliferation of ovarian cancer cells by Cell Counting Kit-8 assay, and the calculation and statistics of value-added rate. b The determination of the effect of different concentrations of drugs on the clone-forming ability of ovarian cancer cells by plate cloning assay. c Calculation and statistics of the number of colony formation (*, $p<0.05$; **, $p<0.01$ vs control group).

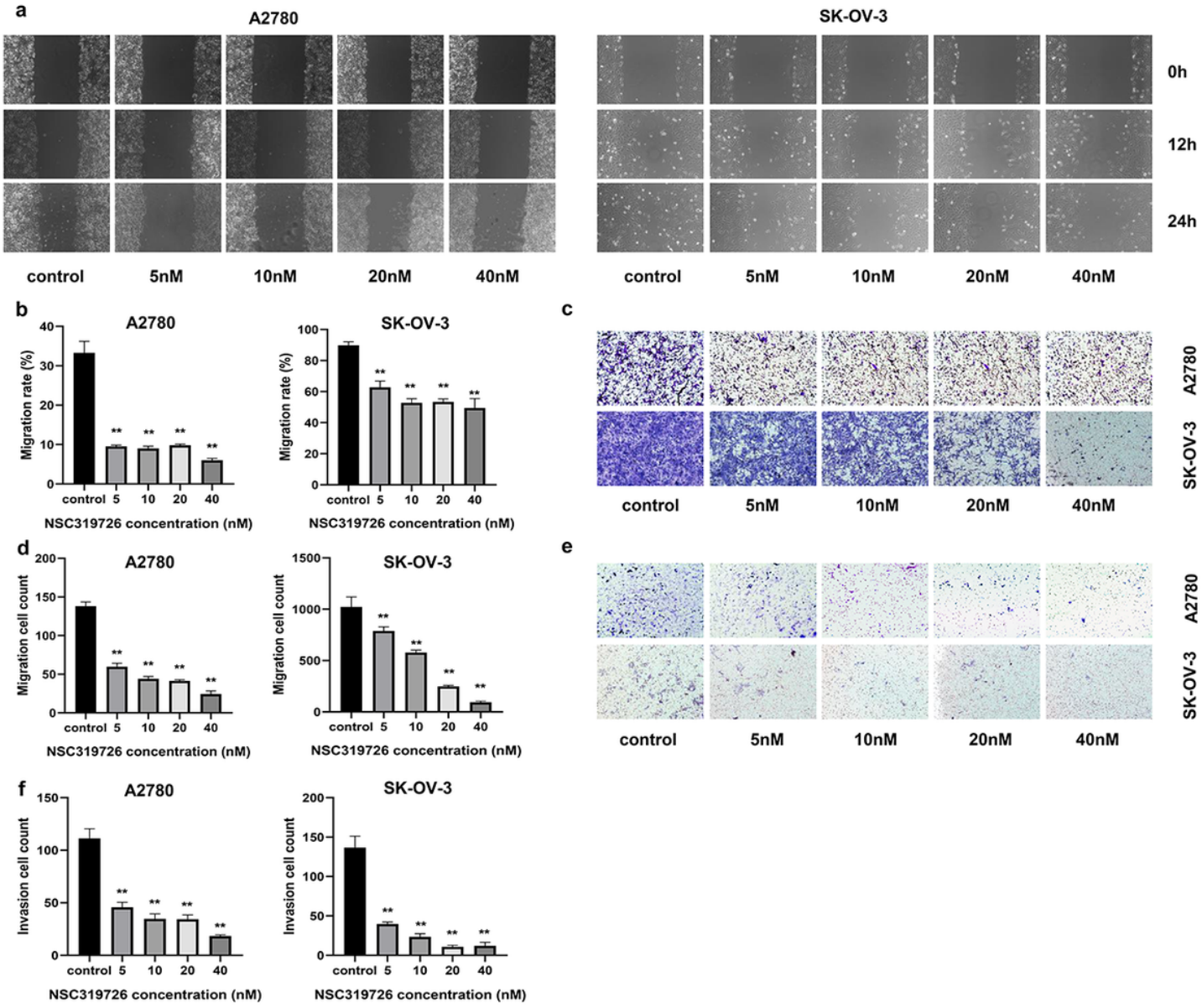

Figure 3

NSC319726 suppresses ovarian cancer cells migration and invasion. a The determination of the effect of different drug concentrations on the migration ability of ovarian cancer cells by Wound healing assay (original magnification 100×). b Calculation and statistics of the migration rate of ovarian cancer cells. c The determination of migration ability of SK-OV-3 and A2780 cells by Transwell assay (original magnification 200×). d Calculation and statistics of the number of migrating ovarian cancer cells. e The determination of invasive ability of ovarian cancer cells by the Invasion assay (original magnification 200×). f Calculation and statistics of the number of invasive cells (*, $p<0.05$; **, $p<0.01$ vs control group).

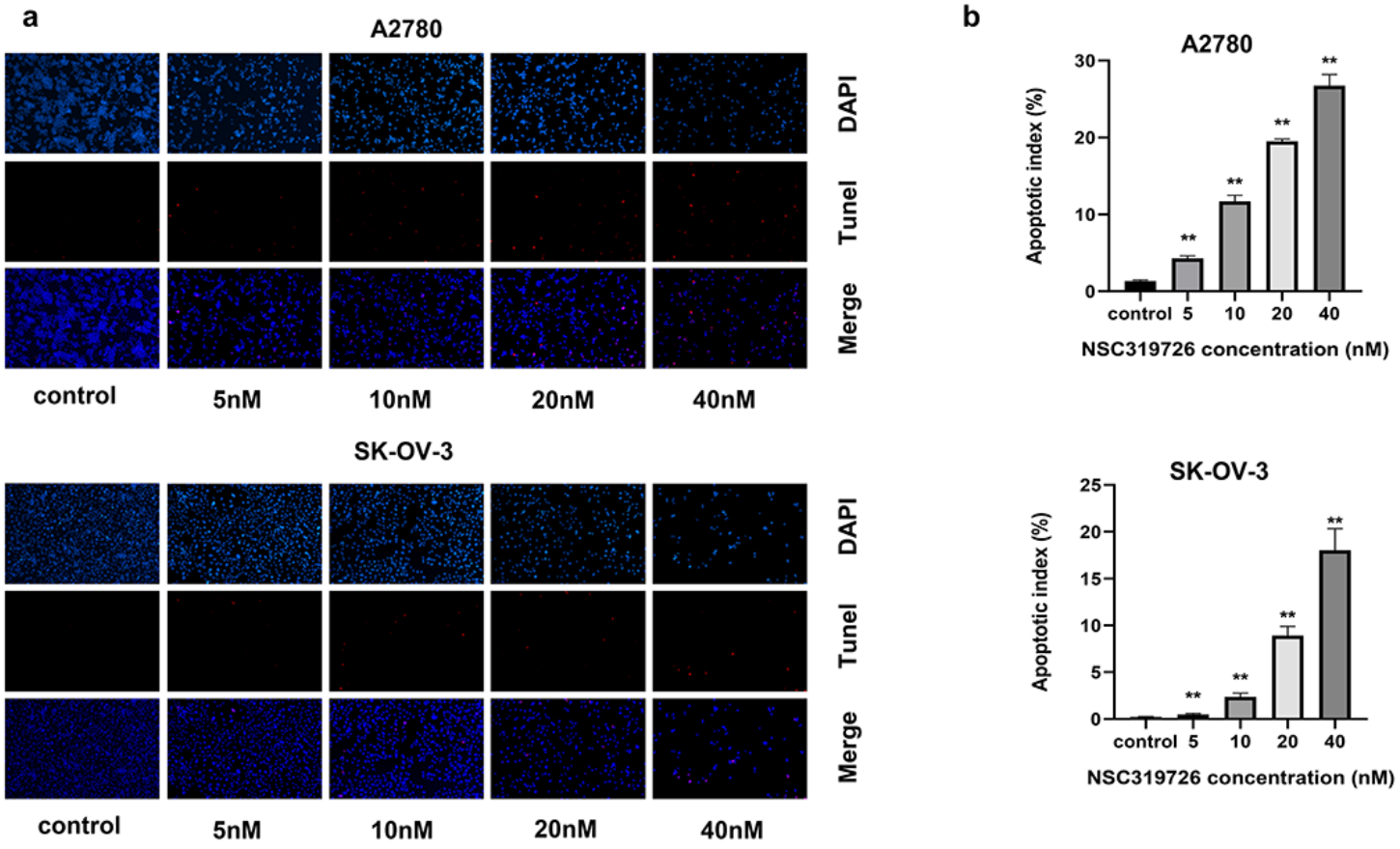

Figure 4

NSC319726 promotes ovarian cancer cells apoptosis. a Detection of apoptosis levels in ovarian cancer cells at different drug concentrations by Tunel staining (original magnification 200×). b Calculation and statistics of the apoptosis rate of ovarian cancer cells (*, $p<0.05$; **, $p<0.01$ vs control group).

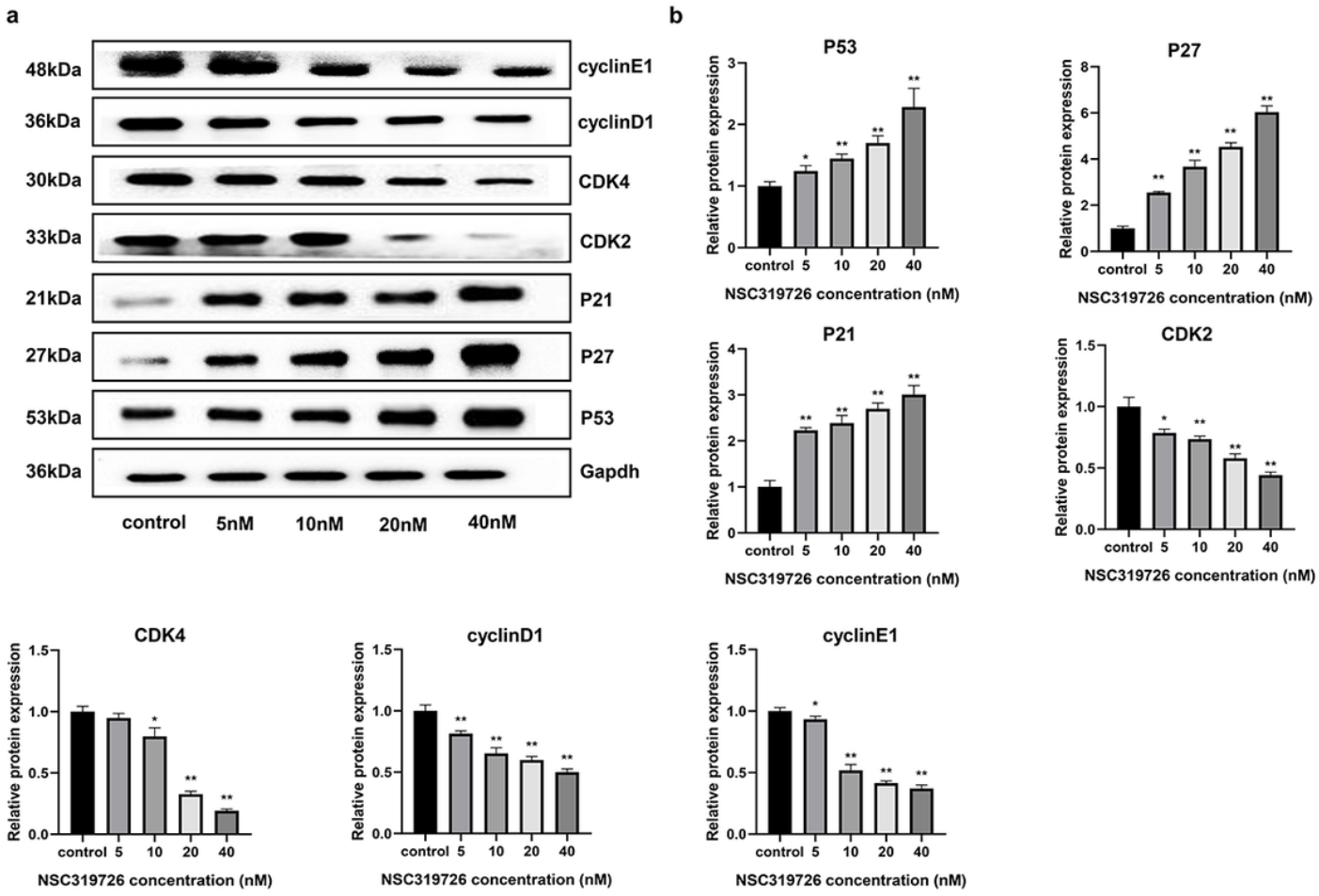

Figure 5

NSC319726 may suppress ovarian cancer cell proliferation through P53-regulated cell cycle arrest. a The detection of expression levels of P53, P27, P21 and cyclin in ovarian cancer cells after treatment with different drug concentrations by Protein immunoblotting assay, with Gapdh as an internal reference control. b Statistical results of protein expression levels involved in (a). (*, $p<0.05$; **, $p<0.01$ vs control group).

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- Supplementaryfile1.xlsx
- Supplementaryfile2.xlsx