

UNBS5162 inhibits SKOV3 ovarian cancer cell proliferation by regulating the PI3K/AKT signalling pathway

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Abstract. Ovarian cancer is the gynaecological malignancy with the highest mortality rate worldwide, and effective and safe therapeutic methods are limited. UNBS5162, a derivative of naphthalimide, has a clear inhibitory effect on the proliferation of various tumour cells *in vitro* and *in vivo* as a pan-antagonist of CXC chemokine ligand expression, but whether it serves a function in ovarian cancer remains unclear. The aim of the present study was to determine the effects of UNBS5162 on the proliferation, migration and invasion of ovarian cancer cells. The cell viability was detected using a Cell Counting Kit-8 (CCK-8) assay. The invasion and migration of SKOV3 cells were determined using Transwell assays. Cell apoptosis was determined using flow cytometry. The apoptosis-associated proteins and associated factors, such as phosphoinositide 3-kinase (PI3K)/protein kinase B (AKT) signalling pathway members, were detected using western blot analysis. The CCK-8 assay revealed that SKOV3 cell viability was affected by UNBS5162 in a dose- and time-dependent manner. In Transwell assays, UNBS5162 inhibited cell invasion and migration. Furthermore, it was identified that UNBS5162 markedly increased the apoptosis rate of SKOV3 cells. Simultaneously, the expression of the anti-apoptotic protein B-cell lymphoma 2 (Bcl-2) was decreased and the expression of the pro-apoptotic proteins active caspase-3 and Bcl-2-associated X protein were increased in SKOV3 cells treated with UNBS5162. In addition, the expression levels of phospho (p-)AKT/total AKT, p-mammalian

target of rapamycin (mTOR)/total mTOR, p-p70 S6 kinase (p70S6K)/total p70S6K and cyclin D1 were decreased in the UNBS5162-treated group. The results of the present study indicated that UNBS5162 inhibits proliferation, migration and invasion, and induces apoptosis in ovarian cancer cells, which may be regulated by the PI3K/AKT signalling pathway. These results suggest that UNBS5162 may be a potential novel drug for clinical ovarian cancer treatment.

Introduction

Ovarian cancer is the gynaecological malignancy with the highest mortality rate, with >120,000 mortalities worldwide annually (1,2). The 5-year survival rate for ovarian cancer is only 30-40% (3). Approximately 70% of patients with ovarian cancer are diagnosed in the later stages of the disease (4). Chemotherapy is a commonly used method for the treatment of ovarian cancer; however, patients with ovarian cancer are likely to develop resistance to chemotherapy drugs owing to continuous use (5). Therefore, development of low toxicity and efficient novel drugs for the treatment of ovarian cancer is urgently required.

Naphthalimide has been a focus of research in the development of antitumour drugs (6,7). Naphthalimides, a class of compounds that bind to DNA by intercalation, exhibit relatively marked anticancer activity against various human cancer cell lines (7,8). Since it has toxic side effects, the primary amine group of naphthalimide has been optimized, and one of the derivatives is UNBS3157 (9). UNBS3157 was designed to avoid the blood toxicity of the clinical metabolite amonafide (10). UNBS3157 is rapidly hydrolysed in physiological saline to produce UNBS5162 which exhibits anticancer activity (11). UNBS5162, a novel type of naphthalimide, was used to avoid the specific effects of the metabolite amonafide, including haemotoxicity (12). UNBS5162 significantly attenuates cell cycle progression of human cancer cells by significantly prolonging the duration of G₂ phase (13). UNBS5162 has been identified to decrease levels of CXC chemokine ligand (CXCL) chemokines, including CXCL1, CXCL5 and CXCL8, in experimental prostate cancer and has a good inhibitory effect on the proliferation of tumour cells *in vitro* and *in vivo* (14). In summary, UNBS5162 has anticancer effects, and can decrease certain side effects. He *et al* (12) identified that UNBS5162

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Abbreviations: CCK-8, Cell Counting Kit-8; PI3K, phosphoinositide 3-kinase; DMEM, Dulbecco's modified Eagle's medium

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inhibits the proliferation of oesophageal cancer squamous cells through the phosphoinositide 3-kinase (PI3K)/protein kinase B (AKT) signalling pathway. Liu *et al* (11) identified that UNBS5162 inhibits the proliferation of human lung cancer cells by promoting cell apoptosis. It has also been identified that UNBS5162 has a therapeutic effect in the treatment of human retinoblastoma (13). However, to the best of our knowledge, UNBS5162 has not been used in the treatment of ovarian cancer.

In the present study, we investigated the effect of UNBS5162 treatment on the proliferation, migration and invasion of ovarian cancer SKOV3 cells, which may be regulated by the enhancement of apoptosis and inhibition of PI3K/AKT.

Materials and methods

Cell lines and cell culture. The ovarian cancer cell line SKOV3 was obtained from the American Type Culture Collection (Manassas, VA, USA). Cells were cultured in Dulbecco's modified Eagle's medium (DMEM; HyClone; GE Healthcare Life Sciences, Logan, UT, USA) supplemented with 10% fetal bovine serum (FBS; Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA), 100 U/ml penicillin (Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) and 0.1 mg/ml streptomycin (Sigma-Aldrich; Merck KGaA) at 37°C in an incubator containing 5% CO₂. When the cells entered the exponential growth phase, they were washed three times with PBS and digested with trypsin (Beijing Solarbio Science & Technology, Co., Ltd., Beijing, China). Following rounding of the cells, DMEM was added to end the digestion, and the cells were treated with DMEM containing 10% FBS repeatedly to obtain a single cell suspension and, finally, the cells were maintained in 6-well plates prior to use in subsequent experiments. A total of 0.1% dimethylsulfoxide (DMSO) was the negative control (NC) group.

Cell Counting Kit-8 (CCK-8) viability assay. SKOV3 cells were digested in 0.25% trypsin solution at room temperature for 2 min and counted to prepare a cell suspension. Subsequently, 96-well plates were seeded at 1,000 cells/well with 100 μ l cell suspension, whereas 0.2, 2, 20 or 200 μ M UNBS5162 was added to the experimental groups. The cells were cultured in a CO₂ incubator as aforementioned, and cell viability was determined every 24 h using the CCK-8 assay (Beijing Solarbio Science & Technology, Co., Ltd.). To each well, 10 μ l CCK-8 reagent was added and cells were incubated at 37°C for 1.5 h. The optical density (OD) values were determined using a microplate reader at 450 nm. Each assay was performed in triplicate.

Cell invasion and migration assays. Matrigel (BD Biosciences, San Jose, CA, USA) was dissolved overnight in serum-free DMEM (diluted 1:6), and 100 μ l was added to the upper chamber of 24-well Transwell plates (EMD Millipore, Billerica, MA, USA). Following shaking evenly, the Matrigel was placed in a CO₂ incubator for 4-6 h at 37°C until gel formation. The culture medium was dried, and 500 μ l serum-free medium was added to the bottom of wells to hydrate the basement membrane for 30 min. The cell suspensions, treated with 20 μ M UNBS5162 for 24 h, were prepared using serum-free DMEM. A 100 μ l

volume of the cell suspension (1x10⁵ cells) was loaded into the upper chamber of the Transwell, and 500 μ l DMEM containing 10% FBS was added to the bottom of Transwell for incubation overnight. The next day, the Transwell was removed, and cells remaining on the upper chamber were removed with a cotton swab. Following washing with PBS, the cells adhering to the membrane were fixed in 4% paraformaldehyde for 30 min at room temperature. Cells were stained with 0.1% crystal violet for 20 min at room temperature. Following washing with PBS, five fields of view were selected randomly under a light and inverted microscope, and images were captured for enumeration of the cells. For the migration assay, the experimental procedure was similar, except that Matrigel was not added to the Transwell chamber, and 5,000 cells were used. Each assay was performed three times independently.

Cell apoptosis assay. Following treatment of SKOV3 cells with 20 μ M UNBS5162 for 24 h, cells were harvested, digested with trypsin without EDTA, centrifuged at 200 x g for 5 min at room temperature and resuspended in pre-cooled PBS at 4°C. The cells were centrifuged once again as mentioned above, and the supernatant was carefully aspirated. Subsequently, 1X binding buffer (Biomiga Inc., San Diego, CA, USA) was added to resuspend the cells, and cells were used at a density of 1-5x10⁶ cells/ml. A 100- μ l aliquot of the cell suspension was transferred into a 5 ml flow tube, stained with 5 μ l annexin V/fluorescein isothiocyanate (FITC) (Beijing 4A Biotech Co., Ltd., Beijing, China) for 5 min at room temperature and kept in a dark place. The samples were stained with 10 μ l propidium iodide (PI) and 400 μ l PBS, and cells were collected and detected using a flow cytometry. The results were analysed by FlowJo software (version 7.6.3; FlowJo, LLC, Ashland, OR, USA). The assay was performed in triplicate.

Western blot assay. Following treatment of SKOV3 cells with 0.1% DMSO and 20 μ M UNBS5162 for 24 h, proteins were extracted with radioimmunoprecipitation lysis buffer (including protease inhibitor) (Beijing ComWin Biotech Co., Ltd., Beijing, China). Protein concentration was determined using the bicinchoninic acid (Beijing ComWin Biotech Co., Ltd., Beijing, China) method. Proteins were heated at 95°C for 5 min, and ~20 μ g protein/group was added to each well in a vertical electrophoresis tank for SDS-PAGE (10% gel) prior to transfer onto a polyvinylidene difluoride membrane. Following blocking with 5% non-fat milk in Tris-buffered saline containing 0.1% Tween-20 (TBST; Beijing ComWin Biotech Co., Ltd., Beijing, China) at room temperature for 1 h, the membrane was incubated with primary antibodies at 4°C overnight. Primary antibodies were against the following: Rabbit anti-human antibodies against AKT (dilution, 1:1,000; cat. no. 4691; Cell Signalling Technology, Inc., Danvers, MA, USA), phospho (p)-AKT (dilution, 1:1,000; cat. no. 4060; Cell Signalling Technology, Inc.), mammalian target of rapamycin (mTOR; dilution, 1:1,000; cat. no. 2983; Cell Signalling Technology, Inc.), p-mTOR (dilution, 1:1,000; cat. no. 5536; Cell Signalling Technology, Inc.), p70 S6 kinase (p70S6K; dilution, 1:1,000; cat. no. 14485-1-AP; ProteinTech Group, Inc., Chicago, IL, USA), p-p70S6K (dilution, 1:1,000; cat. no. 9204; Cell Signalling Technology, Inc.), cyclin D1 (dilution, 1:1,000; cat. no. 60186-1-Ig; ProteinTech Group,

Inc.), B-cell lymphoma 2 (Bcl-2; dilution, 1:1,000; cat. no. 12789-1-AP; ProteinTech Group, Inc.), Bcl-2-associated X protein (Bax; dilution, 1:1,000; cat. no. 23931-1-AP; ProteinTech Group, Inc.), pro-caspase-3 (dilution, 1:1,000; cat. no. 12742; Cell Signalling Technology, Inc.), active caspase-3 (dilution, 1:1,000; cat. no. 25546-1-AP; ProteinTech Group, Inc.) and α -tubulin (dilution, 1:5,000; cat. no. 11224-1-AP; ProteinTech Group, Inc.). Following incubation, the membrane was washed three times in Tris-buffered saline containing 0.1% Tween-20 for 5 min each, and then incubated with horseradish peroxidase-labelled goat anti-rabbit antibody (dilution, 1:5,000; cat. no. SA00001-2; ProteinTech Group, Inc.) secondary antibodies at room temperature for 1 h. Following washing, an enhanced chemiluminescence (ProteinTech Group, Inc.) chromogenic substrate was added to visualize the bands. The grey value was determined using Quantity One software (version 4.62; Bio-Rad Laboratories, Inc., Hercules, CA, USA). The expression of each protein was calculated relative to tubulin. Each blot was performed three times.

Statistical analysis. Results were analysed using Student's t-test or analysis of variance with Fisher's least significant difference post hoc test with SPSS statistical analysis software (version 18.0; SPSS, Inc., Chicago, IL, USA). Results are expressed as the mean \pm standard deviation. $P < 0.05$ was considered to indicate a statistically significant difference.

Results

UNBS5162 inhibits SKOV3 ovarian cancer cell proliferation. A CCK-8 assay was used to determine the effect of UNBS5162 on SKOV3 ovarian cancer cell viability. The viability of SKOV3 cells significantly decreased following 20 and 200 μ M UNBS5162 treatment in a dose-dependent manner ($P < 0.01$ vs. NC; Fig. 1A). Therefore, in subsequent experiments, 20 μ M UNBS5162 was used, as it had a significant and similar inhibitory effect compared with 200 μ M UNBS5162 and lower general toxicity. UNBS5162 decreased the number of SKOV3 cells in a time-dependent manner, with a significantly decreased viability compared with the NC at 72 h ($P < 0.05$; Fig. 1B). These results suggested that UNBS5162 could effectively inhibit the viability of the ovarian cancer cell line SKOV3.

UNBS5162 inhibits SKOV3 ovarian cancer cell invasion and migration. The effects of UNBS5162 on the invasion and migration of the ovarian cancer cell line SKOV3 were investigated using Transwell assays. The number of crystal violet-stained cells in the invasion and migration assays was decreased by UNBS5162 (Fig. 2A). These results were revealed to be significant ($P < 0.05$; Fig. 2B and C), suggesting that UNBS5162 inhibits migration and invasion of ovarian cancer cells.

UNBS5162 promotes SKOV3 cell apoptosis. The effect of UNBS5162 on SKOV3 cell apoptosis was determined using an annexin V/FITC and PI double-staining assay. The apoptosis rate in the UNBS5162-treated group was significantly higher compared with the NC group (24.46% vs. 5.75%;

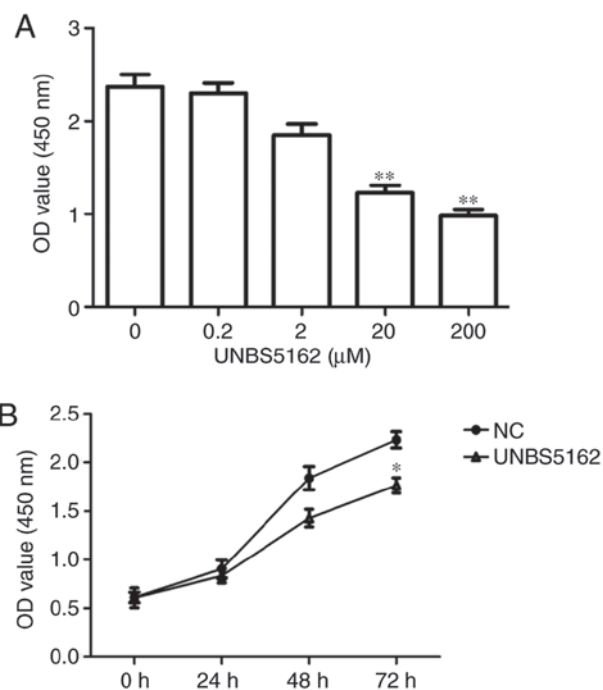


Figure 1. Effect of UNBS5162 on SKOV3 cell proliferation determined using a Cell Counting Kit-8 assay. (A) SKOV3 cells were treated with UNBS5162 (0, 0.2, 2, 20 or 200 μ M) for 72 h. (B) UNBS5162 inhibited SKOV3 cell proliferation in a time-dependent manner. * $P < 0.05$, ** $P < 0.01$ vs. the NC group. NC, negative control; OD, optical density.

$P < 0.05$; Fig. 3A). Furthermore, apoptosis regulators, such as the anti-apoptotic protein Bcl-2, the pro-apoptotic protein active caspase-3 and Bax, were analysed by western blotting (Fig. 3B). Consistent with the flow cytometric results, the expression of the anti-apoptotic protein Bcl-2 decreased, and the expression of the pro-apoptotic protein active caspase-3 and Bax increased in the UNBS5162-treated group compared with the NC group ($P < 0.05$, Fig. 3D). These results indicated that UNBS5162 promotes SKOV3 cell apoptosis.

UNBS5162 suppresses the PI3K/AKT pathway in SKOV3 ovarian cancer cells. The PI3K/AKT signalling pathway is an important signalling pathway in tumours. The mTOR, p70S6K and cyclin D1 proteins were selected as indicators to determine the activity of the PI3K/AKT signalling pathway following UNBS5162 treatment. The western blot results indicated that the p-AKT/total AKT and p-mTOR/total mTOR expression ratios were decreased significantly in UNBS5162-treated SKOV3 cells compared with the NC ($P < 0.05$; Fig. 4). Similarly, the p-p70S6K/total p70S6K expression ratio and cyclin D1 expression level decreased following UNBS5162 treatment ($P < 0.05$; Fig. 4). These results suggested that the proliferation of UNBS5162-treated SKOV3 cells is suppressed via the PI3K/AKT signalling pathway.

Discussion

In the present study, UNBS5162, a derivative of naphthalimide, was investigated to determine its antitumour effect and the underlying molecular mechanism. Specifically, it was identified that UNBS5162 inhibited SKOV3 cell proliferation,

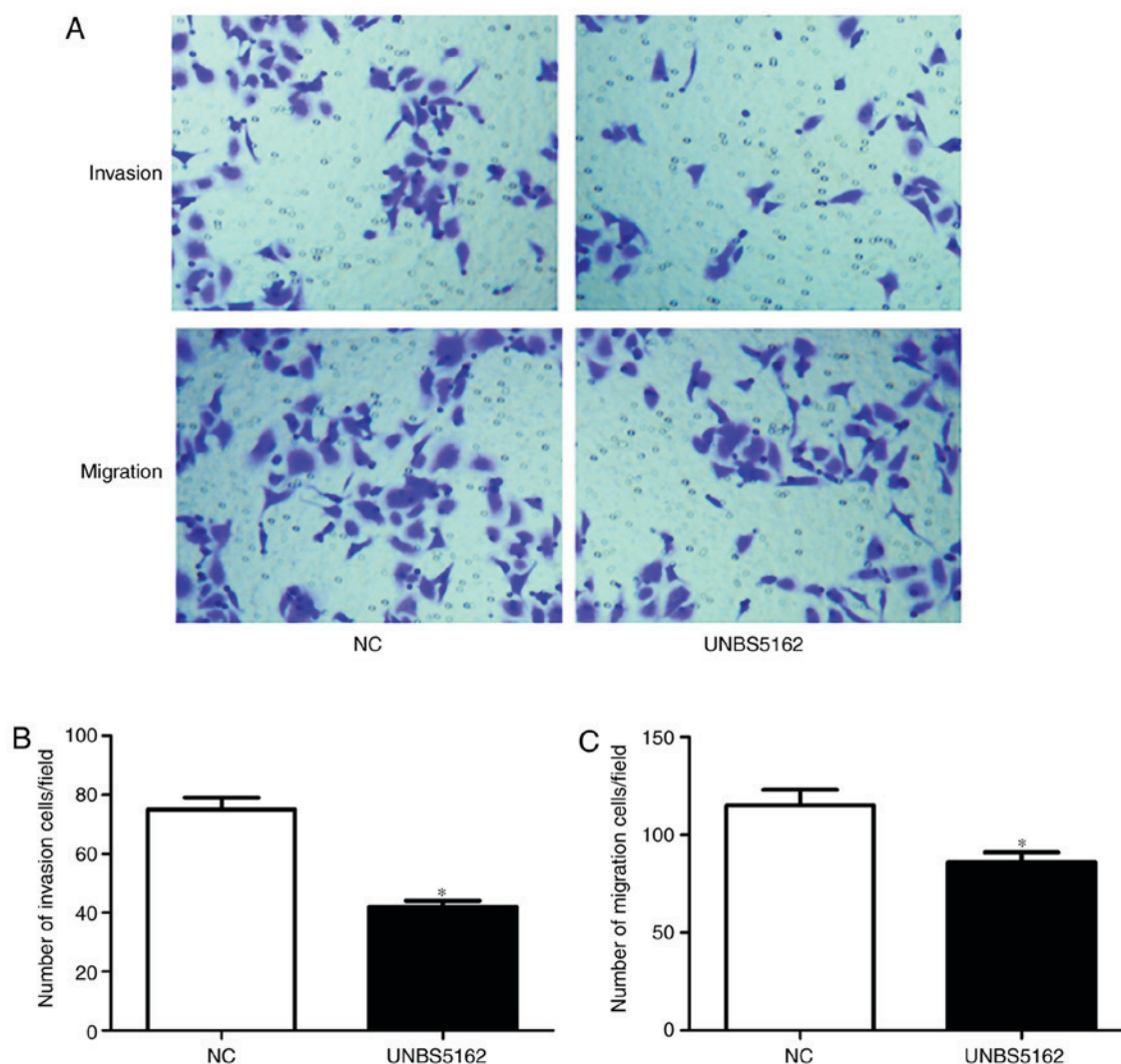


Figure 2. UNBS5162 inhibits SKOV3 cell invasion and migration. (A) Crystal violet-stained invasive and migratory SKOV3 cells were captured using an inverted microscope at x100 magnification. Quantification of (B) invasive and (C) migratory cells. * $P < 0.05$ vs. the NC group. NC, negative control.

invasion and migration. Furthermore, it was identified that cell apoptosis was promoted by UNBS5162. Finally, the effects of UNBS5162 on SKOV3 were identified to be associated with the inhibition of the PI3K/AKT signalling pathway.

Owing to the difference in structure of DNA in normal cells and tumour cells, DNA is an ideal target for anticancer drugs (15). DNA intercalators serve an important function in the treatment of tumours. Naphthalimide compounds are DNA intercalators that can insert into the middle of a base pair, causing changes in the topology of the DNA, and thus affecting topoisomerase function and recognition of DNA (8,16). The naphthalimide derivatives, including amonafide (17), mitonafide (18) and UNBS5162 (19), remain in clinical research. However, in clinical trials, mitonafide and amonafide exhibited marked side effects on the central nervous system, leading to thrombocytopenia, anaemia and leukopenia, and thus these two drugs failed to enter Phase III clinical trials (20). In addition, the clinical effects of these two drugs on solid cancers are limited. To decrease further the toxic side effects by removing the primary amine groups, a novel naphthalimide derivative, UNBS3157, was developed which did not exhibit the blood toxicity of amonafide (9,10). In a previous

study, UNBS3157 was hydrolysed in physiological saline to produce a novel compound that accounts for its anti-cancer activity, and the product was named UNBS5162 (21). However, limited research has been performed in using UNBS5162 on human ovarian cancer.

The results of the present study indicated that UNBS5162 affects the expression of apoptosis-associated proteins. The results of western blot analysis indicated that the expression of Bcl-2 decreased, and the expression of Bax and active caspase-3 increased in the UNBS5162-treated group. These three proteins are well-characterized regulators of apoptosis (22). Bcl-2 is an anti-apoptotic protein, whereas Bax and active caspase-3 are pro-apoptotic proteins (23). Apoptosis is an important antitumour mechanism, and a number of antineoplastic agents exert their function in cancer via apoptosis (24). Studies have indicated that oestrogen-induced apoptosis can be used to treat and prevent breast cancer (25). Shukla *et al* (26) reported a number of applications of apoptosis in prostate cancer. Similarly, UNBS5162 could inhibit A549 non-small cell lung cancer cell proliferation by promoting apoptosis (11). In the present study, it was identified that 20 μ M UNBS5162 exhibited a marked and similar inhibitory

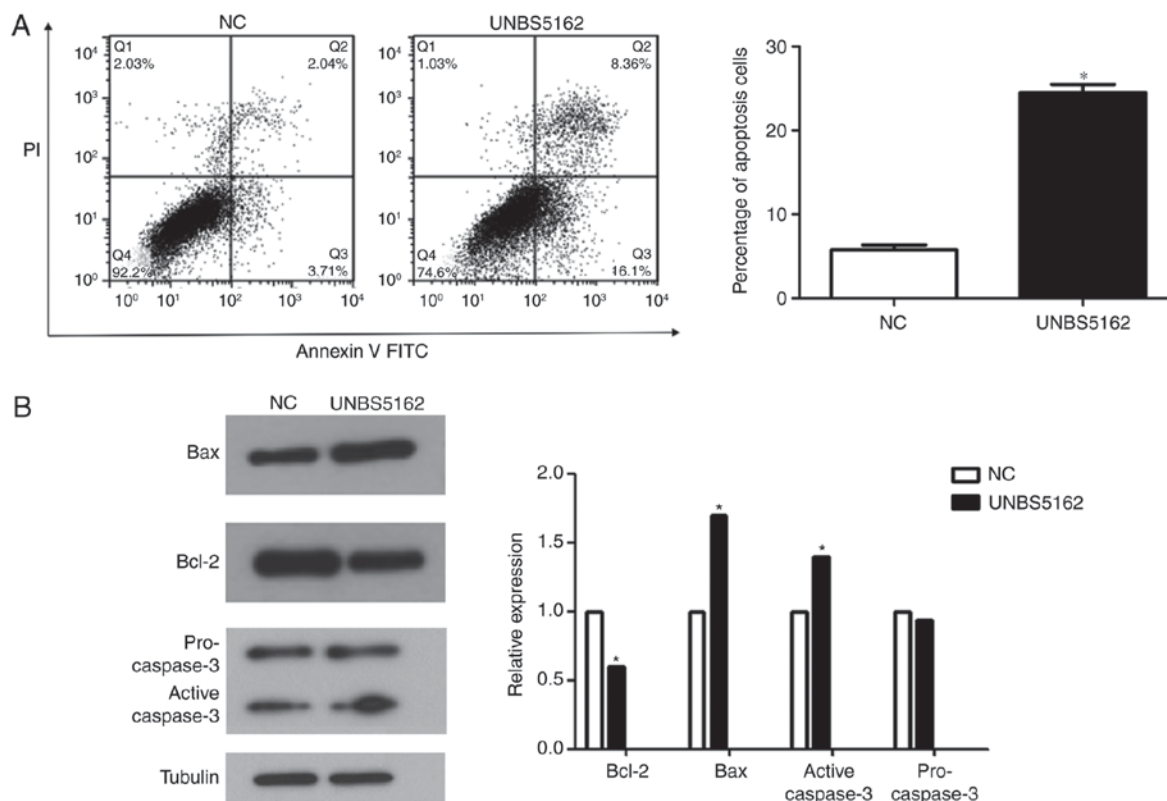


Figure 3. UNBS5162 induces SKOV3 cell apoptosis. (A) SKOV3 cells, treated with UNBS5162 for 24 h, were labelled with annexin V/FITC and PI and analysed by flow cytometry. The proportion of apoptotic cells was determined. (B) Western blot analysis of SKOV3 cells treated with UNBS5162 for 24 h. The band intensities were quantified relative to tubulin expression. * $P < 0.05$ vs. NC group. NC, negative control; PI, propidium iodide; FITC, fluorescein isothiocyanate; Bcl-2, B-cell lymphoma 2; Bax, Bcl-2-associated X protein.

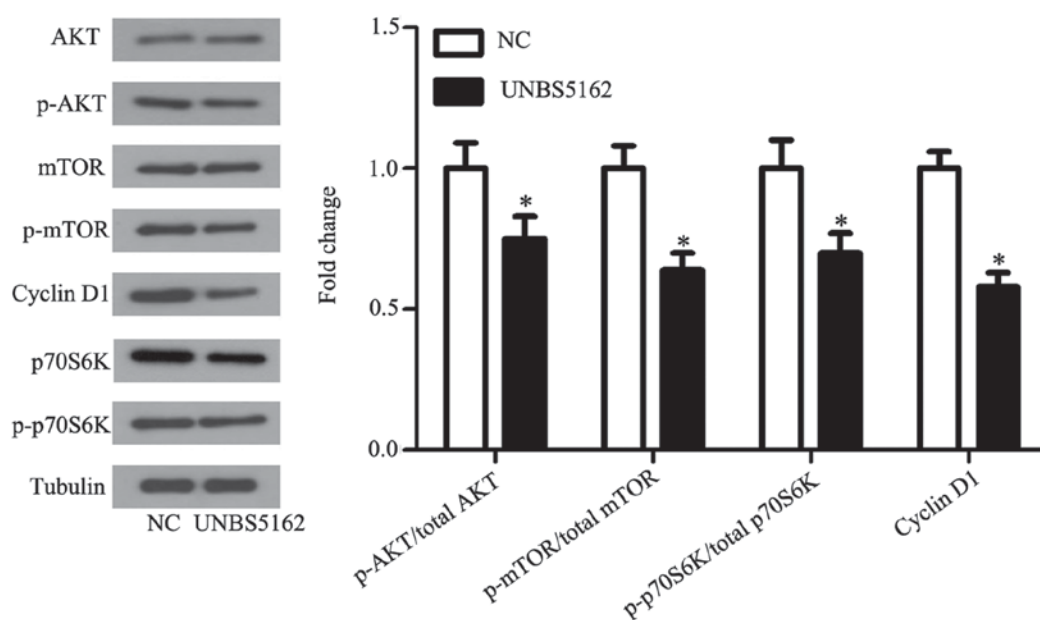


Figure 4. Effects of UNBS5162 on the phosphoinositide 3-kinase/AKT signalling pathway in SKOV3 cells. Expression levels of AKT, p-AKT, mTOR, p-mTOR, p70S6K, p-p70S6K and cyclin D1 were determined in the UNBS5162-treated and untreated SKOV3 cells using western blot analysis. The relative protein levels of p-AKT/total AKT, p-mTOR/total mTOR, p-p70S6K/total p70S6K and cyclin D1 were quantified. * $P < 0.05$ vs. the NC group. AKT, protein kinase B; p-, phospho-; mTOR, mammalian target of rapamycin; p70S6K, p70 S6 kinase; NC, negative control.

effect on, and low toxicity towards, SKOV3 ovarian cancer cells. The activation of apoptosis is regulated by a number of signalling pathways, among which the PI3K/AKT signalling

pathway is important (27). PI3K, as a member of the lipid kinase family, serves a major function in the regulation of apoptosis, angiogenesis, cellular metabolism, senescence and

other processes (28). The PI3K/AKT signalling pathway was identified to be the most frequently affected cancer pathway at the genetic level (29). The PI3K/AKT signalling pathway has a function in ovarian cancer (30). The key protein mTOR, which is a downstream kinase of the PI3K/AKT signalling pathway, has an important function in the proliferation, migration and invasion of cancer cells (31). Previous studies also have indicated that p70S6K and cyclin D1 are located downstream of the PI3K/AKT signalling pathway, which is associated with cell proliferation (32,33). The results of the present study are consistent with those of these previous studies, indicating that UNBS5162 targets key components of the PI3K/AKT signalling pathway, which may be important in the successful treatment of ovarian cancer.

The present study was performed to elucidate the underlying molecular mechanism of the effect of UNBS5162 in ovarian cancer; however, it may have certain limitations. For instance, the use of other cell lines, inhibition such as using short interfering RNA to interfere with the PI3K/AKT signalling pathway and the impact of other signalling pathways are being assessed in our laboratory. The lack of data on non-cancerous cells used as a negative control is also a limitation of the present study. In addition, *in vivo* experimental verification is required.

In summary, the results of the present study indicate that UNBS5162 could inhibit ovarian cancer cell proliferation, invasion and migration, and promote cell apoptosis, potentially through the PI3K/AKT signalling pathway. The results of the present study suggest the possibility of further clinical applications of UNBS5162 in the treatment of ovarian cancer.

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Availability of data and materials

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

QW and WS conceived and designed the study, performed the study, analysed and interpreted the data, and wrote the paper.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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