

UNBS5162 inhibits proliferation of human melanoma cells by inducing apoptosis via the PI3K/Akt pathway

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Abstract. UNBS5162, a novel naphthalimide, is generated by UNBS3157 hydrolysis in physiological saline. In the present study, the effects of UNBS5162 on M14 human melanoma cells were evaluated by Cell Counting Kit-8 and transwell assays, as well as western blotting. The underlying mechanism of apoptosis induced by UNBS5162 was investigated. The results demonstrated that proliferation of UNBS5162-treated M14 melanoma cells was markedly inhibited in a time-dependent manner. The flow cytometry results indicated a markedly increased apoptosis rate in the experimental group compared with in the control group (23.8 ± 0.4 vs. $7.62 \pm 0.5\%$). Microscopy analysis revealed that the invasive and migratory abilities of UNBS5162-treated M14 cells were markedly suppressed. Furthermore, UNBS5162 treatment led to decreased expression of the anti-apoptotic protein B-cell lymphoma 2, but increased expression of the pro-apoptotic proteins Bcl-2-associated X protein and caspase-3. In addition, the expression of several key proteins involved in the phosphatidylinositol-4,5-bisphosphate 3-kinase/protein kinase B/mammalian target of rapamycin (PI3K/Akt/mTOR) signaling pathway was altered in M14 cells treated with UNBS5162. Based on these results, it may be hypothesized that UNBS5162 suppresses the proliferation of M14 cells by inducing apoptosis via inhibition of key proteins in the PI3K/Akt/mTOR signaling pathway.

Introduction

Melanoma is a malignant tumor caused by excessive proliferation of abnormal melanocytes and most commonly occurs in the skin, but also occurs in the mucosa and eye choroid. In addition, melanoma accounts for a large proportion of cases of

skin tumour-associated mortality (1). There are several factors that can induce the onset of melanoma, including sunlight exposure, family history, occurrence of nevus, giant congenital melanocytic nevi and dysplastic nevi syndrome (2,3). In addition, the morbidity of melanoma varies with race and geographical region; for example, the incidence in Caucasians is much higher compared with in people of African descent. Although the survival rate for patients with melanoma has been greatly improved due to advances in research and treatment, the overall morbidity of melanoma is still increasing with an annual increase of 3 to 5% (4,5). Since melanoma cells possess a high degree of malignancy, rapid growth rate and early metastatic characteristics, finding effective treatments to suppress proliferation of these cells is particularly important.

Naphthalimides, a class of compounds that binds DNA by intercalation, are known as potential anti-cancer agents (6). Numerous clinical trials have been performed using naphthalimides, including mitonafide, DMP840, amonafide and elinafide (7). However, dose-limiting bone marrow toxicity, leading to thrombocytopenia, anaemia and leukopenia, has meant that naphthalimides in general and amonafide in particular have failed to pass Phase III clinical trials (8). Amonafide is metabolized by N-acetyl transferase-2 to form N-acetyl amonafide, which is a toxic metabolite (9,10). To prevent toxicity and improve therapeutic outcome, a class of bis-intercalating agents were derived from naphthalimides (11). UNBS3157, a naphthalimide derivative, was designed to overcome the metabolism that induces the clinical haematotoxicity of amonafide. Several studies have reported that UNBS3157 possesses a three- to four-fold higher maximum tolerated dose regardless of route of administration and does not induce blood toxicity at therapeutic doses (12-14).

UNBS5162, N-{2-[2-(dimethylamino)ethyl]-1,3-dioxo-2,3-dihydro-1H-benzo[de] isoquinolin-5-yl}urea, is a novel naphthalimide derivative that can be generated by UNBS3157 hydrolysis in physiological saline (15). The anti-cancer activity of UNBS5162 was investigated by Mijatovic *et al* (16) using experimental models of refractory human prostate cancer and the results revealed that expression of proangiogenic (C-X-C motif) ligand (CXCL) chemokines was almost completely eliminated following UNBS5162 treatment. However, the anti-tumor effects of UNBS5162 in other diseases remain unknown. The aim of the present study was to investigate the

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effects of UNBS5162 on melanoma and its overall mechanism of action.

Materials and methods

Cell culture. The M14 human melanoma cell line was obtained from the Cell Bank of the Chinese Academy of Sciences (Shanghai, China). Cells were maintained in RPMI-1640 (Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) supplemented with 100 U/ml penicillin, 0.1 mg/ml streptomycin (Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) and 10% fetal bovine serum (Invitrogen; Thermo Fisher Scientific, Inc.). Cells were cultured in a humidified atmosphere containing 5% CO₂. Once cell confluence reached ~80%, they were treated with 10 μ M UNBS5162 (MedChemExpress, Princeton, NJ, USA) or 0.1% dimethyl sulfoxide (DMSO, Amresco, LLC, Solon, OH, USA) for 24 h at room temperature. DMSO-treated cells were used as the negative control (NC) group.

Western blotting. Total protein was extracted from UNBS5162- and DMSO-treated cells using radioimmunoprecipitation assay buffer (Beijing ComWin Biotech Co., Ltd., Beijing, China), followed by centrifugation at 12,000 \times g and 4°C for 10 min. Protein concentration was determined using bicinchoninic acid assay and equal amounts of proteins (20 μ g/lane) were separated by 8-15% SDS-PAGE. Following electrophoresis, the proteins on the gel were transferred onto polyvinylidene difluoride membranes. Membranes were blocked with 5% bovine serum albumin (Gibco; Thermo Fisher Scientific, Inc.) for 1 h at room temperature and incubated overnight at 4°C with appropriate primary antibodies purchased from Wuhan Sanying Biotechnology (Wuhan, China), including rabbit anti-human Akt (cat. no. 60203-2-Ig; 1:1,000), phosphorylated (p)-Akt (cat. no. 66444-1-Ig; 1:1,000), mTOR (cat. no. 20657-1-AP; 1:1,000), p-mTOR (cat. no. 20984-1-AP; 1:1,000), rabbit anti-human ribosomal protein S6 kinase (p70S6K; cat. no. 66638-1-Ig; 1:1,000), B-cell lymphoma 2 (cat. no. 60178-1-Ig; Bcl-2; 1:1,000), Bcl-2-associated X protein (Bax; cat. no. 60267-1-Ig; 1:1,000), active caspase-3 (cat. no. 66470-2-Ig; 1:1,000) and GAPDH (cat. no. 60004-1-Ig; 1:5,000). The membranes were subsequently washed using Tris-buffered saline with 0.1% Tween-20 and incubated with goat anti-mouse horseradish peroxidase (HRP)-conjugated IgG secondary antibodies (cat. no. SA00001-1; 1:5,000; Wuhan Sanying Biotechnology, Wuhan, China) or goat anti-rabbit HRP-conjugated IgG secondary antibodies (cat. no. SA00001-2; 1:5,000; Wuhan Sanying Biotechnology) at room temperature for 1 h. Finally, the immunoreactive bands were visualized by the ECLTM Prime Western Blotting system (APG Bio Ltd., Shanghai, China). Semi-quantitative analysis was conducted using Quantity-One software 4.0 (Bio-Rad Laboratories, Inc., Hercules, CA, USA) to measure densitometric values for each band, with GAPDH selected as the internal control. The relative protein expression between target protein and internal control was calculated.

Cell proliferation assay. Cell proliferation was measured using a Cell Counting Kit-8 (CCK-8; Dojindo Molecular Technologies, Inc., Kumamoto, Japan) assay. M14 cells were seeded into 96-well plates at 1,000 cells/well and treated with

10 μ M UNBS5162 or 0.1% DMSO for 1.5 h at 37°C. CCK-8 reagent was added every 24 h and incubated for 1.5 h at 37°C. The optical density (OD) values of each well were measured by a microplate reader set at 450 nm.

Transwell migration and invasion assays. A total of 100 μ l Matrigel (diluted 1:6 in serum-free medium) was added to evenly cover 24-well transwell inserts, and incubated at 37°C for 4-6 h until set. The transwell chamber was washed with serum-free medium. UNBS5162- or DMSO-treated cells (1 \times 10⁵, 100 μ l) were suspended in serum-free medium and added to the upper chambers. RPMI-1640 medium containing 10% fetal bovine serum (500 μ l) was added to the lower chambers, and the cells were incubated overnight at 37°C. Residual cells in the upper chamber were removed with a cotton swab. Cells that passed through the Matrigel-coated membrane were fixed with 4% paraformaldehyde for 30 min and then stained with 0.1% crystal violet for 20 min at room temperature. Cell invasion was quantified using an inverted light microscope (magnification, \times 100; CKX41; Olympus Corporation, Tokyo, Japan) and by randomly selecting five regions for cell counting. The migration assay was conducted following the same protocol as the invasion assay, but without application of Matrigel. A total of 5,000 cells were added to the upper chambers.

Detecting cell apoptosis by flow cytometry. After 24 h of UNBS5162 or DMSO treatment, cells were cultured in serum-free medium for 24 h at 37°C. Subsequently, cells were dissociated using a trypsin solution without EDTA and centrifuged at 2,500 \times g for 5 min at 4°C. Cells were resuspended in 100 μ l Annexin V-fluorescein isothiocyanate (FITC) and propidium iodide (PI) solution (BestBio, Shanghai, China), and incubated at 25°C in the dark for 15 min. After staining, the incubation buffer was removed by centrifugation at 2,500 \times g for 5 min at 4°C and cells were further cultured at 4°C for 20 min in the dark with occasional agitation. Flow cytometry was conducted with an excitation wavelength of 488 nm and the data were analyzed using a FlowJo software 7.2 (Tree Star, Inc., Ashland, OR, USA). In the bivariate scatter plots, necrotic, advanced apoptotic, early apoptotic and live cells are represented in the upper left (Q1), upper right (Q2), lower right (Q3) and lower left (Q4) quadrants, respectively.

Statistical analysis. SPSS 18.0 statistical analysis software (SPSS, Inc., Chicago, IL, USA) was used to analyze the experimental data. All data are expressed as the means \pm standard deviation of three independent experiments. The Student's t-test was used to compare data derived from two independent groups and $P < 0.05$ was considered to indicate a statistically significant difference.

Results

UNBS5162 inhibits proliferation of M14 cells. CCK-8 reagent was used to measure the effects of UNBS5162 on M14 cell proliferation, and the results of the CCK-8 proliferation assay are shown in Fig. 1. UNBS5162 treatment inhibited proliferation of M14 cells in a time-dependent manner. A statistically significant difference ($P < 0.05$) in proliferation between the

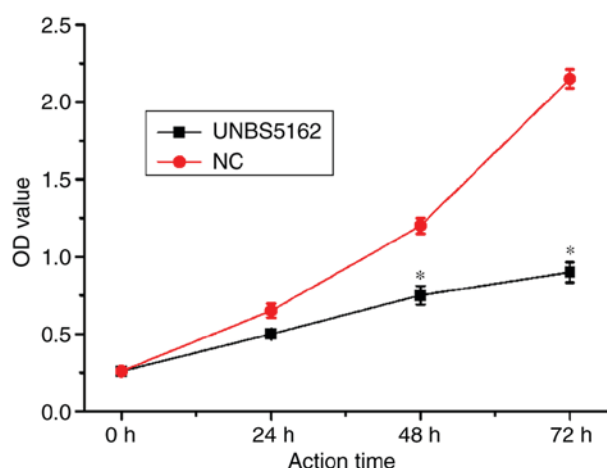


Figure 1. Effects of UNBS5162 on proliferation of human melanoma cells. Cell Counting Kit-8 cell proliferation assays were performed on M14 cells treated with UNBS5162 for 24, 48 and 72 h. * $P < 0.05$ vs. the NC group. NC, negative control; OD, optical density.

UNBS5162- and DMSO-treated groups was observed at 48 and 72 h post-treatment. These results suggested that proliferation of M14 cells may be markedly decreased following prolonged exposure to UNBS5162.

UNBS5162 induces apoptosis of M14 melanoma cells. To investigate whether UNBS5162 causes cell death via apoptosis, Annexin V-FITC/PI staining and flow cytometric analysis were performed. Q2 and Q3 were used to compare the apoptotic rate of M14 cells in the experimental and control group. As shown in Fig. 2A, the apoptotic rate in the Q2 and Q3 quadrants of the NC group was ~4 and 3%, whereas a higher apoptotic rate of ~13 and 11% was observed for cells treated with UNBS5162. These findings demonstrated that total apoptotic rate of UNBS5162-treated cells was increased ($23.8 \pm 0.4\%$) compared with DMSO-treated cells ($7.62 \pm 0.5\%$). In addition, western blotting was utilized to analyse the effects of UNBS5162 on expression of melanoma-associated apoptosis regulators in M14 cells. Western blot analysis of apoptosis regulators following UNBS5162 and DMSO treatment is shown in Fig. 2B. The results revealed a decrease in the expression levels of the anti-apoptotic protein B-cell lymphoma 2 (Bcl-2) and an increase in the expression of proapoptotic proteins, Caspase-3 and Bcl-2-associated X protein (Bax). The expression levels of apoptosis-associated proteins relative to GAPDH are shown in Fig. 2C. Compared with in the NC group, expression of the anti-apoptotic protein Bcl-2 was significantly downregulated, whereas proapoptotic proteins Caspase-3 and Bax were significantly upregulated in the experimental group.

UNBS5162 inhibits migration and invasion of M14 cells. The migration and invasion of UNBS5162- and DMSO-treated cells were observed by microscopy, and the results are shown in Fig. 3. The number of cells counted in five random fields per chamber was reduced in the experimental group compared with in the NC group (Fig. 3A), indicating that the number of migrated (25 ± 6 vs. 57 ± 3 cells) and invasive (62 ± 2 vs. 120 ± 5 cells) M14 cells was significantly reduced following

UNBS5162 treatment ($P < 0.05$; Fig. 3B). Conversely, a large number of cells treated with DMSO was observed in both the migration and invasion assays, as shown in Fig. 3A and B. The results revealed that the invasion and migration of UNBS5162-treated M14 cells was inhibited.

UNBS5162 suppresses activation of the PI3K signaling pathway in M14 cells. The PI3K signaling pathway is considered to be an important signaling pathway in tumours, and the associated proteins Akt and mTOR have a key role in promoting proliferation and metastasis of tumour cells. In addition, p70S6K, a downstream molecule of the PI3K signaling pathway, has been associated with proliferation of M14 cells (17). Western blot analysis was used to evaluate the protein expression levels of Akt, mTOR and p70S6K in M14 cells following UNBS5162 treatment, and the results are shown in Fig. 4A and B. The results revealed a significant downregulation of phosphorylated (p)-Akt and p-mTOR. In addition, the expression levels of p70S6K, a protein associated with cellular proliferation, were significantly downregulated in the experimental group compared with in the NC group. These results suggested that UNBS5162 treatment may suppress PI3K/Akt/mTOR signaling in M14 melanoma cells.

Discussion

The novel naphthalimide, UNBS5162, has been demonstrated to be a DNA-targeting compound that is synthesized by UNBS3157 hydrolysis in physiological saline. UNBS3157 was initially designed to avoid the metabolism that induces haematotoxicity of amonafide (12). It has previously been demonstrated that UNBS3157, a non-haematotoxic naphthalimide, possesses significant anti-cancer activity *in vivo* (13). Therefore, a better understanding of the efficacy and mechanism of action of UNBS5162, a UNBS3157 derivative, is required. Mijatovic *et al* (16) reported that repeated administration of UNBS5162 *in vivo* can markedly improve survival in orthotopic human prostate cancer models by reducing the expression of CXCL chemokines. However, research on the anti-cancer activity of UNBS5162 for other diseases is still lacking; therefore, the aim of this study was to investigate the effect of UNBS5162 on melanoma.

In the present study, a CCK-8 assay was used to measure the proliferation of M14 cells treated with UNBS5162, and the results demonstrated that proliferation was significantly inhibited at 48 and 72 h post-UNBS5162 treatment compared with in the NC group. Furthermore, the results from the transwell assays revealed that the number of invasive and migrated M14 cells was reduced following UNBS5162 treatment, suggesting that UNBS5162 inhibits invasion and migration of M14 cells. Notably, the apoptotic rate of UNBS5162-treated cells was significantly increased, which may have led to reduced invasive and migratory ability. Similar inhibitory effects in human retinoblastoma cells have been previously reported (18).

It has been reported that certain intracellular proteins can regulate apoptosis, including Bcl-2, Bax and Caspase-3 (19,20). Bcl-2, an anti-apoptotic factor, is closely associated with tumour occurrence and resistance (21). Bax, a member of the proapoptotic protein family, when overexpressed not only allows various cell types to undergo spontaneous apoptosis,

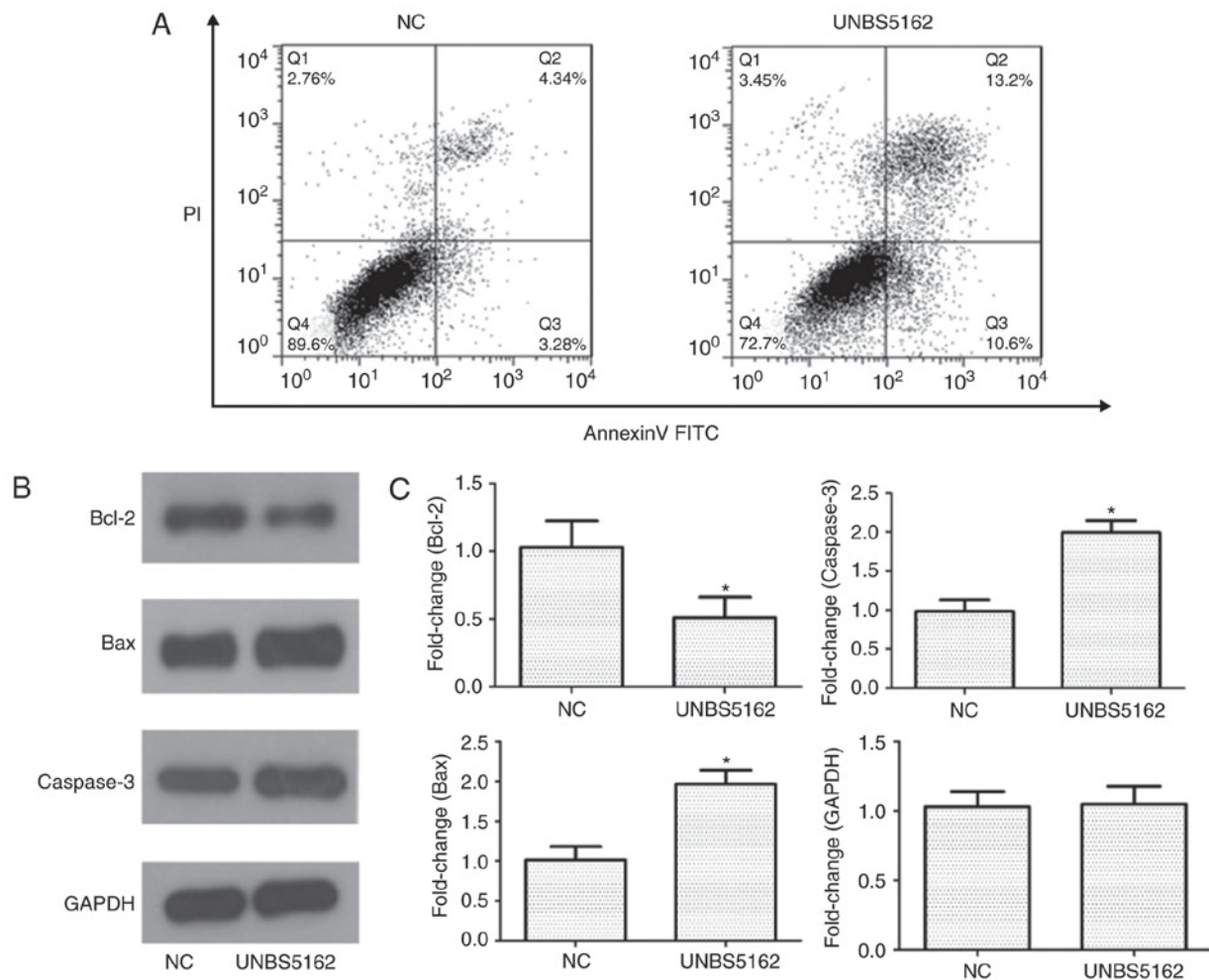


Figure 2. Detection of M14 cell apoptosis following UNBS5162 treatment. (A) Representative flow cytometry scatter plots of cells stained with Annexin V-FITC and PI. (B and C) Bcl-2, Bax and Caspase-3 protein expression levels in M14 cells were detected by western blotting. Results are representative of three independent experiments performed in triplicate. * $P < 0.05$ vs. the NC group. Bax, Bcl-2-associated X protein; Bcl-2, B-cell lymphoma 2; FITC, fluorescein isothiocyanate; NC, negative control; PI, propidium iodide.

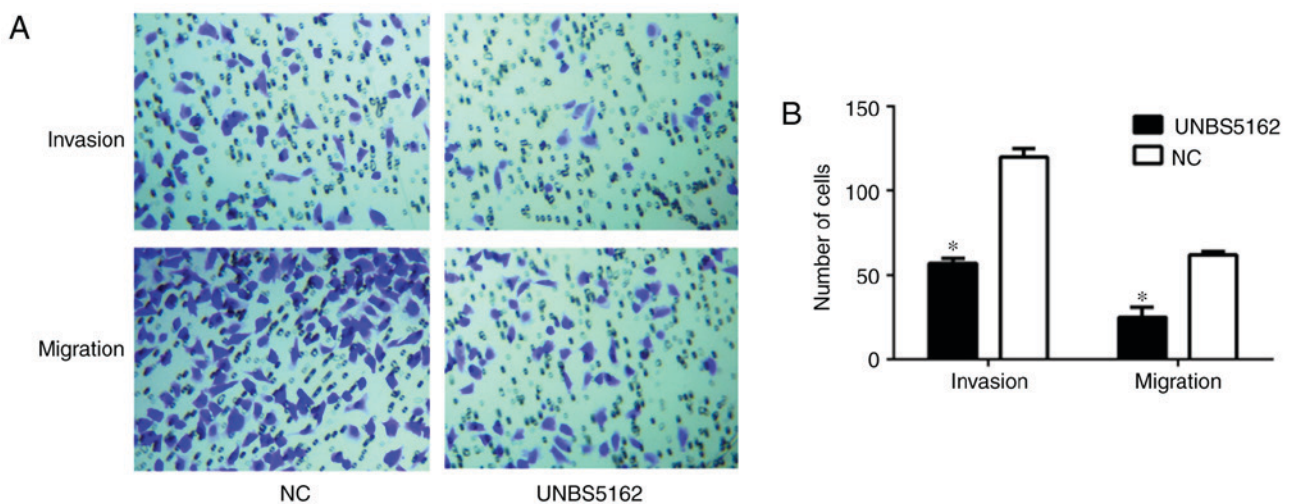


Figure 3. UNBS5162 inhibits M14 cell migration and invasion. (A) Migration and invasion of UNBS5162-treated M14 cells was determined using transwell migration and invasion assays, and visualized under a microscope. Magnification, $\times 100$. (B) The number of invasive and migrated cells was quantified. The average number of cells was quantified from three independent experiments. * $P < 0.05$ vs. the NC group. NC, negative control.

but also promotes apoptosis induced by other factors (22,23). Notably, the proteins Bcl-2 and Bax are antagonistic, and have

a role in apoptosis via formation of a heterodimer (24,25). Furthermore, Caspase-3 is required for Bax-induced apoptosis,

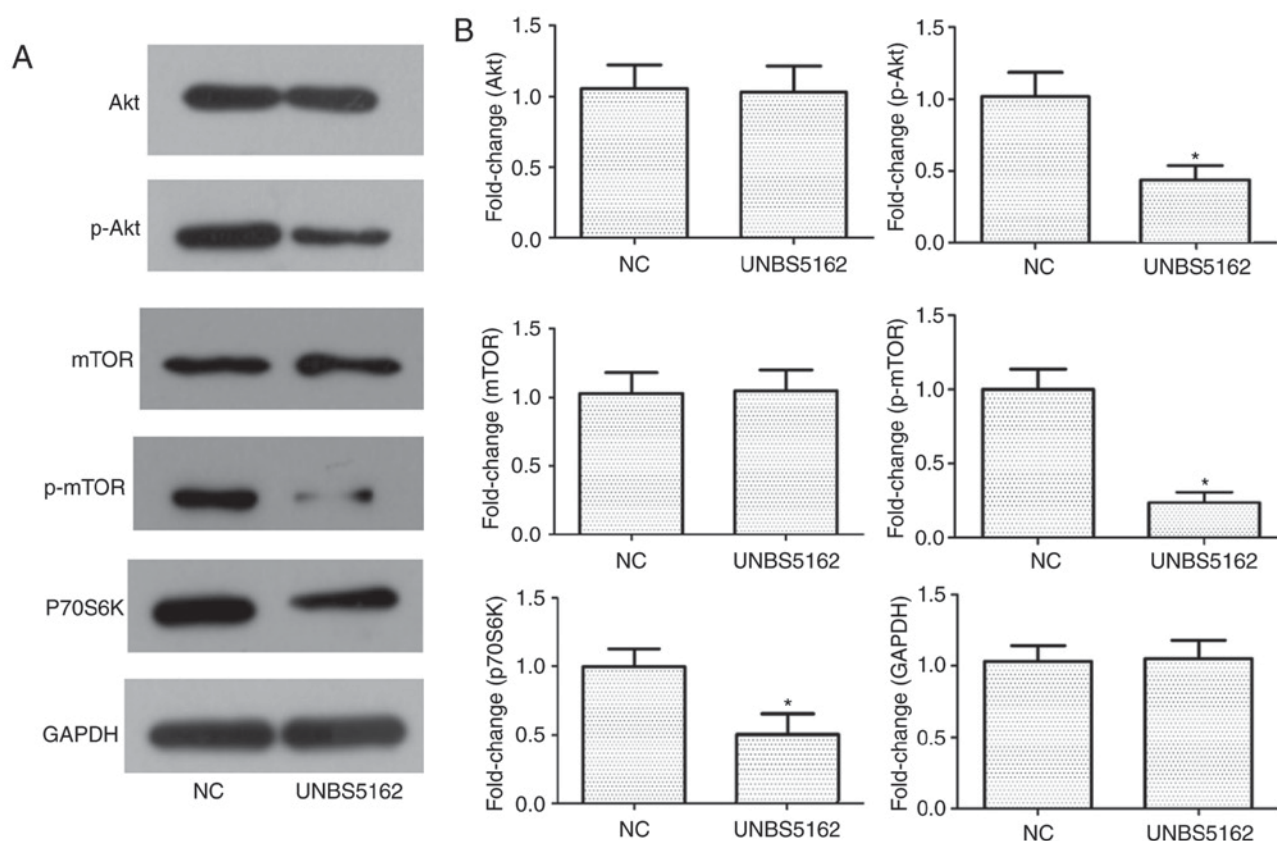


Figure 4. Activation of the PI3K/Akt signaling pathway is inhibited in UNBS5162-treated M14 cells. (A) Expression levels of Akt, p-Akt, mTOR, p-mTOR, p70S6K and internal control GAPDH in M14 cells were determined by western blot analysis. (B) Semi-quantitative analyses of protein expression levels are shown. Results are presented as the means \pm standard deviation from three independent experiments. * $P < 0.05$ vs. the NC group. Akt, protein kinase B; mTOR, mammalian target of rapamycin; NC, negative control; p70S6K, p70 ribosomal protein S6 kinase; p-, phosphorylated; PI3K, phosphatidylinositol-4, 5-bisphosphate 3-kinase.

and is located downstream of the Bcl-2 protein family. Therefore, activation of Caspase-3 and its role in apoptosis is directly affected by the ratio of Bcl-2 and Bax expression (26). In the present study, the western blotting results demonstrated that expression of the anti-apoptotic protein Bcl-2 was reduced in M14 cells treated with UNBS5162, whereas the proapoptotic protein Bax was highly expressed. In addition, Caspase-3 was highly expressed in UNBS5162-treated cells. These results suggested that UNBS5162 may contribute to enhancement of apoptosis in M14 cells.

The molecular mechanisms underlying the effects of UNBS5162 on proliferation and apoptosis were also investigated using M14 cells. Apoptosis is a programmed cell death process, which can be triggered by alterations in the intracellular and extracellular environment or by a death signal, and involves activation, expression and regulation of a series of genes. Activation of the PI3K/Akt/mTOR signaling pathway can inhibit stimulation-induced apoptosis and enhance cell cycle progression, and sequentially improve survival and increase proliferation of tumour cells (27,28). PI3K in the PI3K/Akt/mTOR signaling pathway can increase activation of Akt by stimulating a signaling cascade that produces phosphatidylinositol triphosphate (29,30). Akt is generally located at important intersections of multiple signaling pathways and can respond to various of intra- or extracellular stimuli by regulating survival signals (31,32). Furthermore, the serine/threonine-protein kinase mTOR is a downstream effector of the PI3K/Akt signaling pathway, which

acts as a key regulator of metastasis, invasion, survival and proliferation of tumour cells by activating p70S6K (33,34). It has previously been suggested that the expression of Bcl-2 and Bax proteins may be regulated by kinases and that their activation could be affected by mTOR (35).

In the present study, p-Akt and p-mTOR expression was downregulated following treatment of M14 cells with UNBS5162. Similarly, the expression levels of p70S6K, a downstream molecule in the PI3K/Akt/mTOR signaling pathway, were significantly reduced in the experimental group compared with in the NC group. Downregulation of proteins in the PI3K/Akt/mTOR signaling pathway indicated that UNBS5162 treatment may inhibit PI3K/Akt/mTOR signaling in M14 melanoma cells. Additionally, the migratory and invasive abilities of UNBS5162-treated cells were significantly suppressed. Wang *et al* (18) reported that the functional roles of UNBS5162 in human retinoblastoma cells may be regulated by the activity of the Akt-mTOR pathway *in vitro*. Additionally, the preclinical development of UNBS5162 in prostate cancer has been studied by Mahieu *et al* (15); the results indicated that UNBS5162 has anti-cancer effects in prostate cancer. Therefore, UNBS5162 may be a potential therapeutic agent for melanoma.

In conclusion, the results of the present study demonstrated that UNBS5162 significantly inhibits proliferation, invasion and migration of M14 cells. In addition, it may be hypothesized that UNBS5162 induces apoptosis through regulation of the PI3K/Akt/mTOR signaling pathway.

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Availability of data and materials

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

Authors' contributions

XLS performed the experiments and analyzed the data, and was also a major contributor in writing the manuscript. LY, JX, YMZ and JZC made substantial contribution to the study conception and design. ZPL, HYL, XZC and JHF performed the statistical analysis. JHF was involved in the drafting of the manuscript and gave the final approval for publication. All authors read and approved the manuscript.

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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