

UNBS5162 and amonafide inhibits tumor progression in human melanoma by the AKT/mTOR pathway

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Background: Human melanoma is a malignant tumor originated from melanocytes with high invasion, metastasis, and poor prognosis. In this study, the effects of naphthalimides UNBS5162 and amonafide on the properties of proliferation and apoptosis in human melanoma cells were confirmed.

Methods: Cell proliferation was determined by CCK8 and clone formation assay. Transwell assay was performed to detect the migration and invasion of M14 and A375 cells. Cell apoptosis was estimated using flow cytometry.

Results: In a drug sensitivity assay, cell viability decreased with increasing concentrations of UNBS5162 or amonafide. Likewise, proliferation of M14 or A375 cells treated with 10 μ M UNBS5162 or 8 μ M amonafide decreased significantly when compared with negative control (NC) cells, their inhibition effect verified by means of a clone formation assay. After the treatment with UNBS5162 or amonafide, the migration of melanoma cells was inhibited in a dose-dependent manner. The number of invaded cells treated with UNBS5162 was also significantly reduced when compared with those of the NC cells. The apoptotic cell numbers treated with UNBS5162 or amonafide decreased significantly when compared with the M14 and A375 cells in the NC group. According to Western blot results, phosphorylation of AKT and expressions of mesenchymal marker factors were inhibited in cells treated with UNBS5162 or amonafide.

Conclusion: These results reveal that UNBS5162 inhibits the cell activity of melanoma cells through the AKT/mTOR signaling pathway, and reverses epithelial–mesenchymal transition conversion in human melanoma cells. This study on UNBS5162 and amonafide in melanomas provides an experimental basis of their uses and potential value on human melanoma treatment.

Keywords: cell apoptosis, proliferation, M14, A375, epithelial-mesenchymal transition

Introduction

Human melanomas are malignant tumors that originated from melanocytes with high invasion, metastasis, and poor prognosis. Melanomas accounts for about 1%–2% of human malignant tumors, with less than a 90% 5-year survival rate.¹ At initial diagnosis, about 20% of patients with melanomas have tumor metastasis. Unfortunately, there is still a deficiency of effective drugs for melanoma, thus new anti-neoplastic drugs that have fewer side effects are needed.^{2,3} Therefore, our study has aimed to investigate the inhibitory effect of UNBS5162 and amonafide on human melanoma.

As DNA intercalating agents, naphthalimides have high potential as anti-neoplastic agents. Currently, naphthalimides have been evaluated clinically; this includes mitonafide, Elinafide, amonafide, bisnafide, and UNBS5162.^{4,5} According to present research, UNBS5162 and amonafide are promising antitumor agents endowed with fewer side effects and a higher efficiency of cancer suppression.^{6–8} However, the clinical

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efficacy of UNBS5162 and amonafide for human melanoma remains unclear. In this study, we investigated the effects of UNBS5162 and amonafide on the properties of proliferation and apoptosis in humans. Our purpose is to determine the functional mechanism of UNBS5162 and amonafide in human melanomas *in vitro*.

Materials and methods

Cell lines

The human melanoma cell lines M14 (BRAF^{V600E} positive) and A375 (BRAF^{V600E} positive) were purchased from the Type Culture Collection of the Chinese Academy of Sciences, Shanghai, China. Cells were cultured in DMEM containing 10% FBS, streptomycin (100 units/mL), and penicillin (100 units/mL; Invitrogen Corp., Waltham, MA, USA).

Compounds and antibodies

UNBS5162, amonafide, and 5FU were purchased from the American MedChemExpress, Monmouth Junction, NJ, USA. Further, reference antibodies Bcl-2 (proteintech, Rosemont, IL, USA; Cat# 12789-1-AP), active-Caspase3 (Abcam, Cambridge, UK; Cat# ab32042), Bax (proteintech, Cat# 50599-2-Ig), P70/S6K (Abcam, Cat# ab109393), AKT (Abcam, Cat# ab8805), p-AKT (Abcam, Cat# ab38449), mTOR (Abcam, Cat# ab32028), p-mTOR (Abcam, Cat# ab109268), Cyclin D1 (proteintech, Cat# 12363-1-AP), E-cad (Abcam, Cat# ab40772), Snail (proteintech, Cat# 13099-1-AP), N-cad (Abcam, Cat# ab6528), Twist (proteintech, Cat# 11752-1-AP), Slug (proteintech, Cat#) and GAPDH (proteintech, Cat# 60004-1-Ig) were used in this study. Our electrochemiluminescence kit was obtained from proteintech.

Drug sensitivity assay

The cells were seeded in 96-well plates at 1,000 cells/well. Nineteen gradients of UNBS5162 were prepared, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 20, 30, 40, 50, 60, 80, 100, 150, and 200 μ M. Nineteen gradients of amonafide were likewise prepared: 0.5, 1, 1.5, 2, 3, 4, 5, 6, 7, 8, 9, 10, 15, 20, 30, 40, 50, 100, and 150 μ M. After cells were treated with UNBS5162 or amonafide for 24 hours, they were cultured with cell-counting kit-8 (CCK8) reagent (Solarbio Science & Technology Company, Beijing, China) for 2 hours, and the OD value of each well was read at 450 nm. Three duplicates were used for each determination.

Cell proliferation assay

A CCK8 assay was performed to detect the proliferation of melanoma cells. Cells of UNBS5162 group were treated with 10 μ M UNBS5162, amonafide cells were treated with 8 μ M

amonafide, and negative control (NC) cells were treated with 0.1% DMSO. A total of 1,000 cells per well were seeded in a 96-well plate. Cellular activity was measured every 24 hours, adding 10 μ L CCK8 reagent per well and reading the absorbance at a wavelength of 450 nm. Cell proliferation was assessed relative to the NC group, and SD was assessed from triplicates.

Clone formation assay

Melanoma cells were treated with UNBS5162 or amonafide for 24 hours. Cells were then collected and seeded on a 6-cm dish at a total number of 200–300. Cells were continually cultured until clones could be observed by the naked eye. After being stained with 0.1% crystal violet for 20 minutes and washed with PBS twice, HCC cells were imaged and counted.

Transwell assay

Cell migration and invasion were confirmed using transwell chambers (Millipore, Billerica, MA, USA), which was performed according to the manufacturer's protocol. Cells were seeded into the upper chamber of a 24-well invasion assay containing matrigel (BD Biosciences, San Jose, CA, USA). Then, DMEM medium with 10% FBS was added into the lower chamber. After 48 hours incubation, cells that migrated to the lower surface of the chamber were stained with 0.1% crystal violet. Experiments were performed in triplicate.

Western blotting

Total proteins were extracted from M14 or A375 cells by a RIPA buffer containing 0.1% proteinase inhibitor. Proteins were separated on 7% or 10% SDS-PAGE and were transferred to a PVDF membrane. Primary antibodies were used at 1:1,000 dilutions, and secondary antibodies were used at 1:5,000 dilutions at 1 hour incubation. Enhanced chemiluminescent (ECL) developer was ordered from proteintech. Experiments were performed in triplicate.

Flow cytometry analysis

Cells were seeded into a 6 cm plate and treated with 10 μ M UNBS5162 or 8 μ M amonafide, being incubated for 48 hours. Annexin V-FITC/PI was added into the cell suspension, and incubated for 5 minutes in the dark. After staining with 10 μ l dye liquor, the samples were analyzed by a flow cytometer (BD FACSCanto II, BD Biosciences). Apoptosis was analyzed using Flowjo software.

Statistical analyses

Results were expressed as the means \pm SDs. Relative densitometry values were calculated considering GAPDH as an

internal control using ImageJ software and were plotted in GraphPad Prism 7. A *t*-test was performed in order to analyze the changes between each group. All results were tested in three independent experiments. $P < 0.05$ was considered as significant.

Results

UNBS5162 and amonafide inhibits the proliferation of human melanoma cells

Based on previous findings, we predicted that UNBS5162 and amonafide would inhibit melanoma cell proliferation.^{9,10} Drug sensitivity assay was performed in order to confirm our prediction. Human melanoma cells were treated with various concentrations of UNBS5162 or amonafide and cultured with CCK8 reagent in order to determine OD values. As shown in Figures 1(A and B), cell viability decreased with increasing concentrations of UNBS5162 and amonafide, with 10 μ M UNBS5162 and 8 μ M amonafide being chosen as experimental concentration. We then examined the proliferation of the UNBS5162 group (10 μ M UNBS5162), the amonafide group (8 μ M amonafide), and the NC group (0.1% DMSO) in M14 and A375 cells by means of CCK8 assay. We observed that the OD value of M14 and A375 cells treated with UNBS5162 or amonafide was significantly reduced when compared to the NC group (Figure 1C). Clone formation assay was then performed to further verify the effects of UNBS5162 and amonafide on cell proliferation (Figure 1D). Likewise, the clone numbers of M14 or A375 cells treated with UNBS5162 and amonafide both decreased significantly when compared with that of NC cells (Figure 1E). These results proved that UNBS5162 and amonafide prevented the melanoma cells from proliferating in vitro.

UNBS5162 and amonafide inhibits the migration and invasion of human melanoma cells

Transwell assay was performed to investigate the effect of UNBS5162 and amonafide on the migration and invasion of melanoma cells. After treatment for 48 hours, UNBS5162 dose dependently decreased the migration and invasion of M14 and A375 cells (Figures 2A and B). Additionally, amonafide (5–15 μ M) decreased the migration and invasion of M14 and A375 cells when compared with NC cells (Figures 2C and D). These results prove that UNBS5162 and amonafide prevent the migration and invasion of melanoma cells in vitro.

UNBS5162 and amonafide promote apoptosis of melanoma cells in vitro

The role of UNBS5162 and amonafide on apoptosis was confirmed by means of flow cytometry. As shown in Figures 3A and B, the apoptosis cell numbers of melanoma cells treated with UNBS5162 (M14, 25.8%; A375 24.97%) and amonafide (M14, 23.64%; A375, 25.7%) increased significantly when compared with NC cells (M14, 9.31%; A375, 0.6%). Western blot was then performed to investigate the change of expression in apoptotic-related genes in UNBS5162, amonafide, and NC groups (Figures 3C and E). Results demonstrate that Active Caspase3 and Bax expression increased in UNBS5162 and amonafide group cells, while Bcl-2 was inhibited (Figures 3D and F).

UNBS5162 and amonafide inhibits growth of melanoma cells by the AKT/mTOR pathway

According to our knowledge, AKT/mTOR is a crucial signaling pathway in tumorigenesis; this also includes melanomas.^{11,12} However, the role of UNBS5162 and amonafide on the AKT/mTOR pathway in melanoma progression remains unknown. Therefore, we hypothesized that UNBS5162 and amonafide would regulate the signaling pathway and, thus, inhibit tumor progression. To investigate UNBS5162 and amonafide's effect on the AKT/mTOR pathway, we measured the expression of related genes in M14 and A375 cells. As shown in Figures 4A–D, phosphorylation of AKT and mTOR were inhibited in M14 and A375 cells when compared with NC cells. The cells were treated with 10 μ M UNBS5162 or 8 μ M amonafide for 24 hours. Further, we detected expressions of P70/S6K and Cyclin D1, which in the UNBS5162 group and amonafide groups were reduced significantly. These results reveal that UNBS5162 and amonafide influences cell viability of melanoma cells through the AKT/mTOR signaling pathway.

UNBS5162 and amonafide inhibit epithelial–mesenchymal transition (EMT) conversion in human melanoma cells

In outside studies, it was found that UNBS5162 and amonafide inhibited the invasion of melanoma cells. Therefore, we further explore the role of UNBS5162 and amonafide in the EMT process of human melanoma cells by means of Western blot. Our results demonstrated that N-cad, Snail, Vimentin, and slug expression in the UNBS5162 and amonafide groups decreased significantly and that E-cad

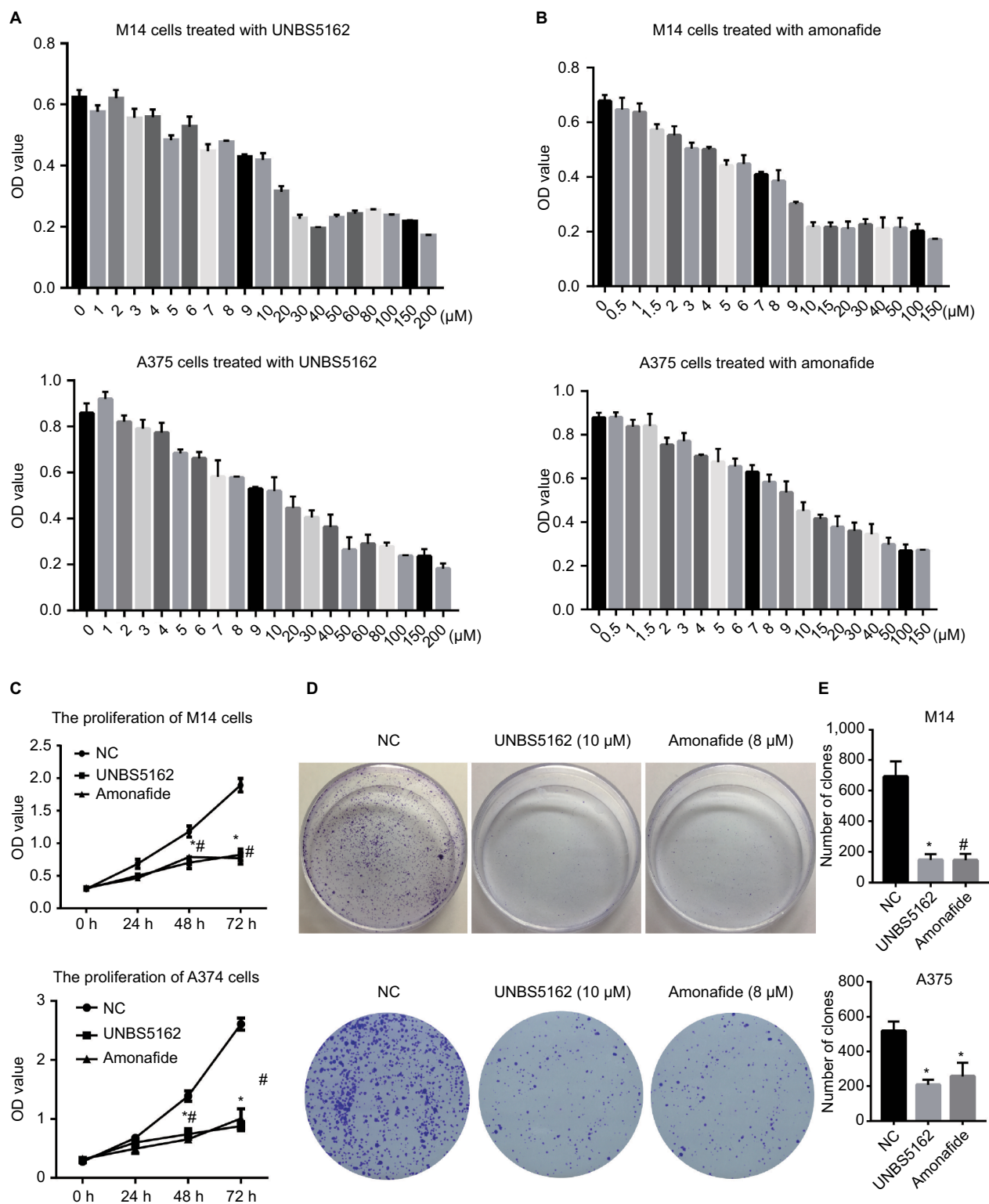


Figure 1 UNBS5162 and amonafide inhibits the proliferation of melanoma cells. (**A** and **B**) The proliferation of M14 or A375 cells treated with various concentrations of UNBS5162 and amonafide, respectively. (**C**) Cell-counting kit-8 assay was performed in order to estimate the proliferation of melanoma cells treated with 10 μM UNBS5162 or 8 μM amonafide; the OD value of the UNBS5162 and amonafide groups decreased significantly compared with the negative control (NC) group both in M14 and A375 cells. (**D** and **E**) Clone numbers of melanoma cells treated with 10 μM UNBS5162 or 8 μM amonafide decreased significantly compared with the NC group.

Notes: * $P < 0.05$, the UNBS5162 group vs the NC group; # $P < 0.05$, the amonafide group vs the NC group.

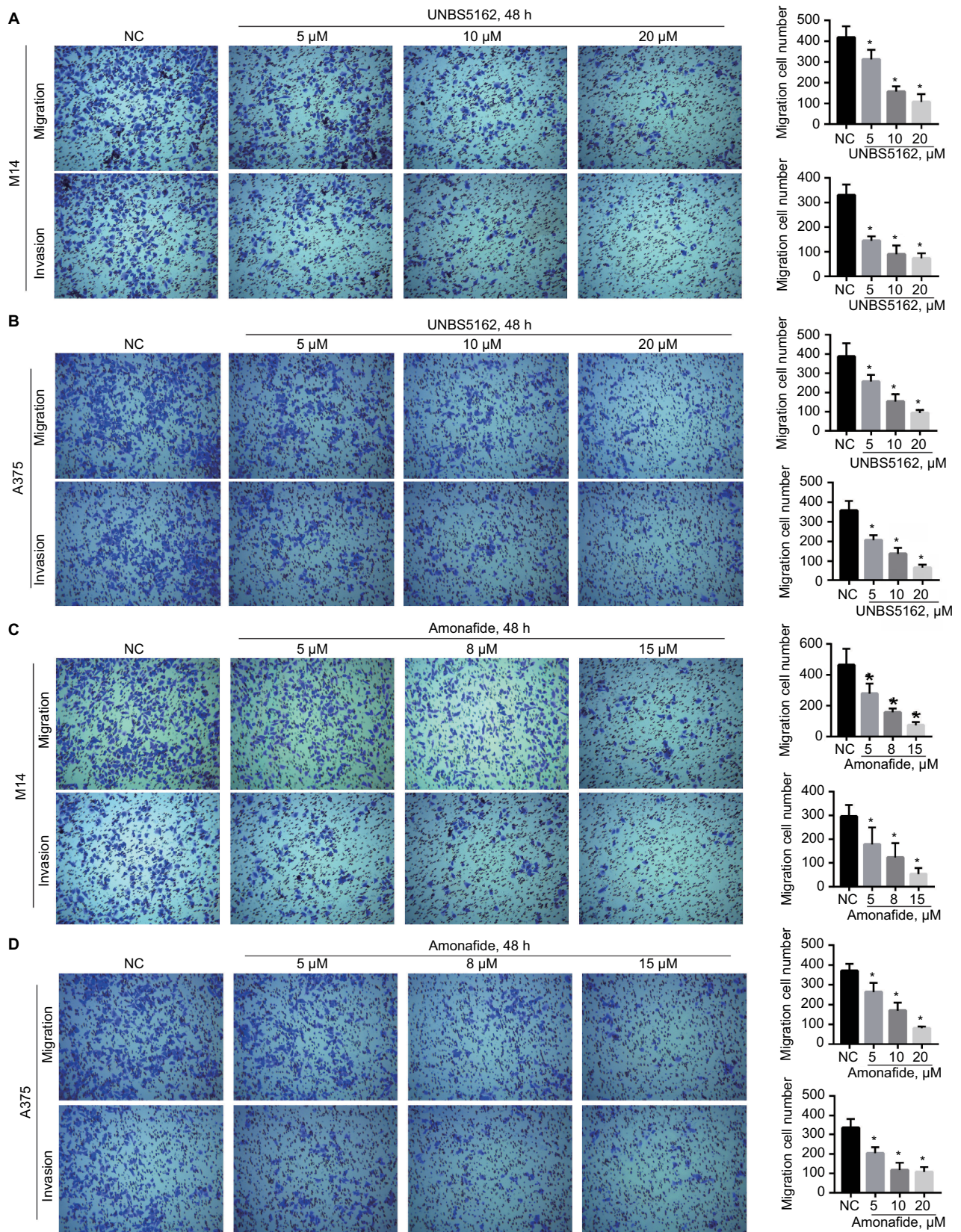


Figure 2 UNBS5162 and amonafide inhibits the migration and invasion of melanoma cells. **(A and B)** Representative images of the transwell assay for migration and invasion in melanoma cells with treatment of UNBS5162. **(C and D)** Representative images of transwell assay for migration and invasion in melanoma cells with treatment of amonafide. Invasion or migration cell numbers decreased significantly in UNBS5162 or amonafide treated cells when compared with negative control (NC) group both in M14 and A375 cells.

Note: * $P < 0.05$.

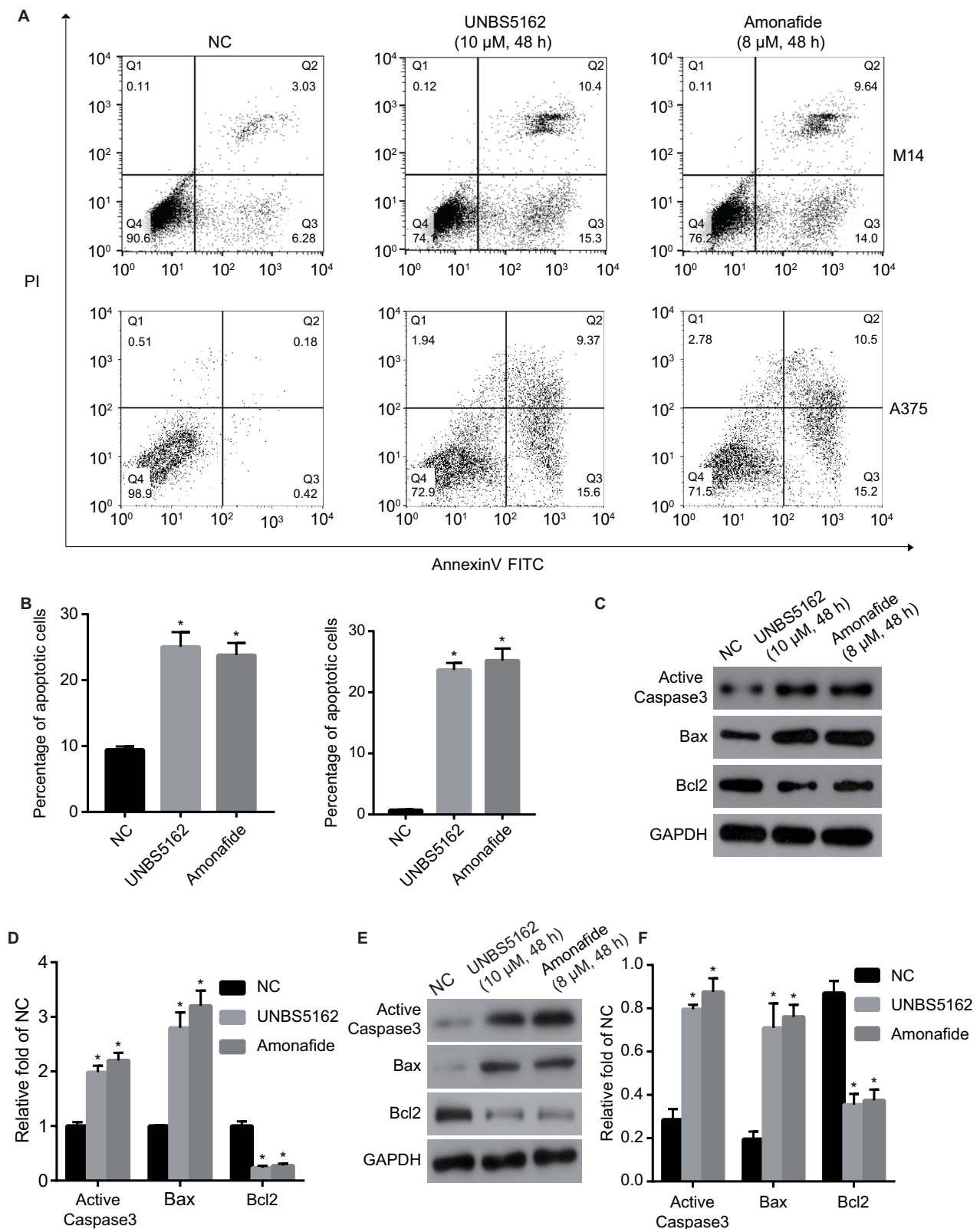


Figure 3 UNBS5162 and amonafide promote the apoptosis of melanoma cells. **(A)** Cell apoptosis was detected by flow cytometry. **(B)** Apoptosis cell number of M14 and A375 cells treated with 10 μ M UNBS5162 or 8 μ M amonafide decreased significantly when compared with negative control (NC) cells. **(C and E)** The expressions of apoptotic-related genes were detected by Western blot. **(D and F)** Active Caspase3 and Bax expression were inhibited in UNBS5162 and amonafide groups, while Bcl-2 was promoted when compared with NC cells.

Note: * $P < 0.05$.

Abbreviations: PI, propidium iodide; FITC, fluorescein isothiocyanate.

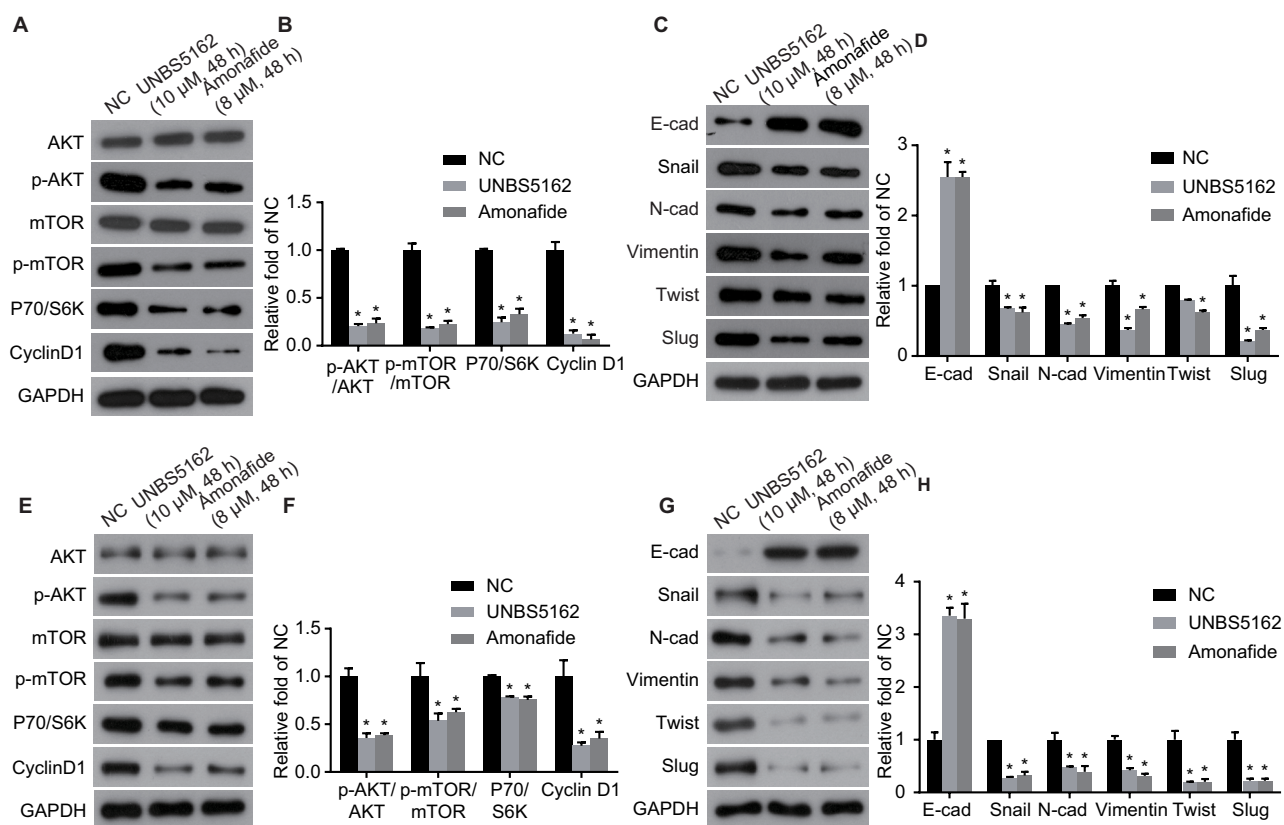


Figure 4 UNBS5162 and amonafide inhibit the activation of the AKT/mTOR pathway and reverse the epithelial–mesenchymal transition process in human melanoma cells. (A–D) Western blot was performed to confirm the pathway by which UNBS5162 and amonafide inhibited tumor progression; phosphorylation of AKT and mTOR were inhibited in M14 and A375 cells treated with 10 μM UNBS5162 or 8 μM amonafide for 48 hours, as well as P70/S6K and Cyclin D1. (C–H) The expressions of N-cad, Snail, Vimentin, and slug in UNBS5162 and amonafide groups decreased significantly, whereas E-cad increased significantly.

Note: * $P < 0.05$.

Abbreviation: NC, negative control.

increased significantly in both groups, whereas Twist in the amonafide group decreased significantly (Figures 4E–H). Thus, our results suggest that UNBS5162 and amonafide reverse EMT conversion in human melanoma cells.

5FU combined with UNBS5162 or amonafide inhibits the proliferation of human melanoma cells

5FU is currently one of the most widely used anticancer drugs in clinical practice,¹³ so we chose it for comparative study. The effects of 5FU combined independently with UNBS5162 or amonafide were tested using a CCK8 assay. These effects were tested in order to measure the proliferation of M14 cells. We found that the CCK8 OD of cells treated with 5 μM 5FU combined with 10 μM UNBS5162 was significantly decreased when compared with cells only treated with 5FU (Figure 5A). Furthermore, similar results were observed in the amonafide-treated cells. The CCK8 OD of M14 and A375 cells decreased when treated with 5FU combined with 8 μM amonafide. This was measured in comparison to 5FU treated cells (Figure 5B).

Discussion

Naphthalimides plays an important role in tumor chemotherapy, and its clinical application has continued to attract researchers.^{14–16} Brana et al¹⁷ first synthesized naphthalimide and its derivatives in 1979, and it has proved effective in being embedded into DNA, increasing the length of DNA, and unwinding superhelix DNA. UNBS5162 and amonafide are two important members of the naphthalimide family, and have recently entered clinical trials.^{18,19} The National Cancer Institute has already evaluated UNBS5162 anti-tumor ability, and has confirmed that it is effective for multiple tumor models. UNBS5162 inhibits tumor progression with less-toxic side effects.²⁰ It was already known that amonafide is also an inhibitor of topoisomerase II (TopoII).

In our present study, we have investigated the role of UNBS5162 and amonafide in melanoma cell growth. A drug sensitivity assay was performed to estimate the change in proliferation of cells treated with UNBS5162 or amonafide. Our results showed that UNBS5162 and amonafide inhibited melanoma cell proliferation, which was consistent with previous results.^{20,21} Next, UNBS5162 and amonafide's effect on

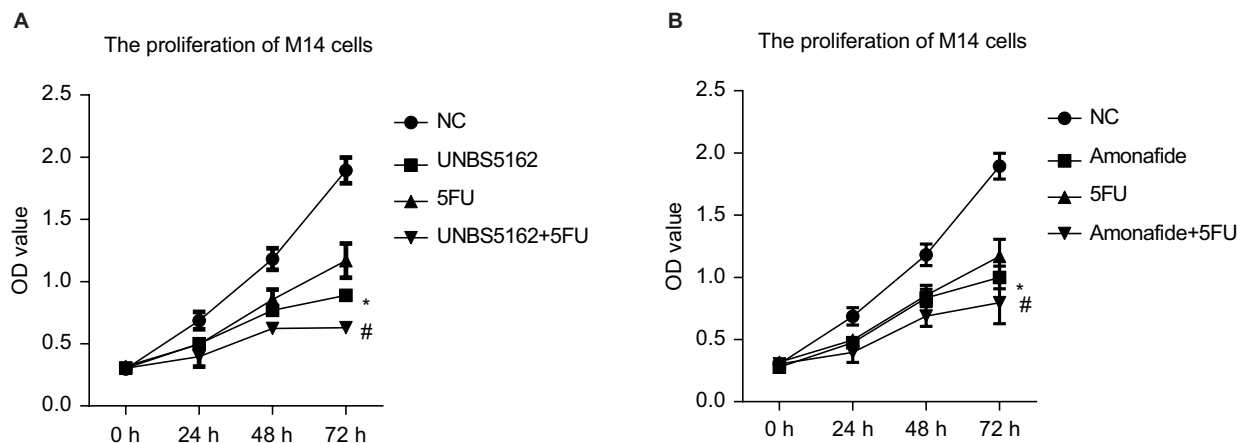


Figure 5 5FU combined with UNBS5162 or amonafide inhibits the proliferation of human melanoma cells. **(A)** CCK8 assay was performed to confirm the proliferation of cells when treated with 5FU combined with 10 μ M UNBS5162. **(B)** CCK8 assay was performed to confirm the proliferation of cells with treatment of combined 5FU and 8 μ M amonafide.

Notes: * $P < 0.05$ vs the NC group; # $P < 0.05$ vs the 5FU group.

Abbreviations: CCK8, cell-counting kit-8; NC, negative control

cell proliferation was verified by CCK8 and clone formation assay. We then estimated the UNBS5162 and amonafide ability of invasion in melanoma cells by means of a transwell. The cells treated with UNBS5162 or amonafide ability of invasion were reduced significantly when compared with NC cells. Flow cytometry was then performed to confirm the role of UNBS5162 and amonafide on apoptosis. The apoptosis cell numbers of the UNBS5162 group and the amonafide group increased significantly when compared with the NC group. Finally, we confirmed the mechanism by which UNBS5162 inhibits progression of melanoma cells. The expression of genes related to the AKT/mTOR signaling pathway decreased significantly when compared with the NC group. Moreover, UNBS5162 and amonafide significantly reverses the EMT conversion in human melanoma cells.

As a naphthalimide, UNBS5162 can reduce the expression of CXCL chemokine in tumors. Chemokines are a class of chemically inducible cytokines that can promote inflammation and can further cause chemotaxis, cell survival and proliferation, intracellular calcium production, and gene transcription.^{22,23} It has also been verified that chemokines are closely related to the occurrence and development of tumors.²⁴⁻³⁰ Furthermore, according to recent findings, CXCLs such as CXCL1, CXCL8, CXCL12, CXCL10, CXCL6, CXCL4, and CXCL2 are all associated with the progression of melanomas.³¹⁻³⁶ Therefore, we predict that UNBS5162 may play a tumor suppressive role in melanomas by means of regulating CXCL. It has been demonstrated that UNBS5162 can induce cancer in PC3 prostate cancer cells. It does this by decreasing expression of Hsp70 by means of the lysosomal membrane permeabilization. Notably, UNBS5162

also decreases the expression of cancer cell's chemoresistance gene HERP in many human tumors.²⁰ Research has confirmed that UNBS5162 is a pan-antagonist of CXCL chemokine expression. When administered alone, UNBS5162 displays antitumor effects in experimental models of refractory prostate cancer; when coadministered with the taxoid, UNBS5162 enhances taxol activity.³⁷ In sum, our data suggested that UNBS5162 and amonafide could inhibit the growth of human melanoma cells by the AKT/mTOR signaling pathway, and thus might have clinical potential in treating melanomas. Further studies of UNBS5162 and amonafide's efficacy and mechanism in vivo and in vitro are needed, however, and will thus be the direction of our future research. They can cause single-strand breaks, double-strand breaks and DNA-protein cross-links in human leukemic cells. Studies have shown that amonafide has a therapeutic effect on advanced breast cancer and leukemia.⁸ Clinical trials of amonafide in a variety of tumors are also in progress.^{21,38}

Furthermore, we proved that combined 5FU and UNBS5162 or amonafide could significantly inhibit the proliferation of melanoma cells in comparison to only 5FU treated cells. The combined effect of two drugs on cell proliferation was significantly stronger than that of each drug alone, which had a synergistic effect. The low-dose combination of two drugs played a synergistic role in anti-proliferation, and also avoided the side effects caused by high-dose drugs on the body, thereby improving the quality of life of patients. However, the specific mechanism of the above effects remains to be further studied.

In conclusion, we demonstrated that UNBS5162 and amonafide could inhibit the growth of human melanoma cells

by AKT/mTOR signaling pathway. The present study on the clinical efficacy of UNBS5162 and amonafide in melanoma provides an experimental basis of its use and expands the potential value on human melanoma treatment.

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Disclosure

The authors report no conflicts of interest in this work.

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