

A LY-15, a novel cyclic pentapeptide that inhibits B16 cell proliferation and migration and induces cell apoptosis

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Abstract. Melanoma is highly resistant to most traditional treatments; therefore, its incidence and mortality rates are rapidly increasing. The effect of a novel sansalvamide A analogue named LY-15 on the growth and induction of apoptosis in B16 cancer cells was investigated *in vitro*. The inhibitory effects of LY-15 on B16 cells occurred in a concentration- and time-dependent manner. The B16 cells were cultured in various concentrations of LY-15 (5, 15 and 25 μ M), and the ameliorating effect of LY-15 was evaluated using apoptotic protein markers B-cell lymphoma 2 (Bcl-2), Bcl-2-associated X protein (Bax), caspase-3 and caspase-9. Furthermore, LY-15 effectively inhibited the B16 cell migration, increased the expressions levels of caspase-3, caspase-9 and the pro-apoptotic Bax, and reduced that of the anti-apoptotic Bcl-2. These findings suggested that LY-15 is a promising chemotherapeutic agent against melanoma by inducing apoptosis through the mitochondrial-associated death pathway. In addition, sansalvamide A analogue LY-15 may a significant therapeutic target for the treatment of malignant melanoma cancer.

Introduction

Malignant melanoma tumor incidence has an annually increasing trend worldwide. Moreover, melanoma accounts for the largest part of skin cancer deaths (1,2). In addition, melanoma is not sensitive to radiotherapy or chemotherapy, and leads a serious problem to clinical treatment. The development of new and highly effective anti-tumor compounds is urgent and challenging. Furthermore, marine microorganisms

and secondary metabolism are promising sources for novel anti-cancer drugs (3). Sansalvamide A, which is a depsipeptide isolated from a marine fungi (*Fusarium spp.*), exhibits anti-tumor activity on multiple cancer cell lines (4,5) and significant anti-proliferative effects in the National Cancer Institute's panel of 60 cancer cell lines (6). Sansalvamide peptide has anti-tumor activities that became the basis for developing a series of cyclic peptide analogues with N-methylation. The synthesis of sansalvamide A analogues received sustained attention in recent years. Brominated sansalvamide peptide inhibits pancreatic cancer cell growth through the G0/G1 cell-cycle arrest (7). Methoxylized zygosporamide peptide inhibits B16 cell growth and induces cell apoptosis (8). Cyclic epi-pentadepis peptide induces differentiation and inhibits proliferation in murine melanoma B16 cells (9). Furthermore, the compound LY-15, which was synthesized by the cyclization of the chain pentapeptide in solution, has a molecular formula and weight of $C_{37}H_{55}N_5O_5$ and 649.42, respectively (Fig. 1). Here, we focused on the effects of LY-15 on the growth and apoptosis of B16 cancer cell lines. Results showed that the compound has greater potency when screened for the growth inhibition of B16 cancer cells, suggesting that LY-15 might be a promising therapeutic agent.

Materials and methods

Materials. The RPMI 1640 and trypsin-EDTA solution were purchased from Gibco; Thermo Fisher Scientific, Inc., (Waltham, MA, USA). The fetal bovine serum (FBS) was purchased from Bovogen Biologicals Pty Ltd., (Keilor East, Victoria, Australia). The Cell Counting Kit-8 (CKK-8) was purchased from Beijing Zoman Biotechnology Co., Ltd., (Beijing, China) and the bicinchoninic acid kit was purchased from Multi Sciences Co., Ltd., (Shanghai, China). The polyvinylidene fluoride (PVDF) membranes were purchased from Roche Applied Science (Penzberg, Germany). The antibody against β -actin (polyclonal rabbit anti-mouse) was purchased from Hangzhou HuaAn Biotechnology Co., Ltd., (Hangzhou, China). The antibodies against B-cell lymphoma 2 (Bcl-2), Bcl-2-associated X protein (Bax), caspase-3 and caspase-9 (all polyclonal rabbit anti-mouse) were purchased from Arigo Bio (Taiwan, Xinzhu). The secondary fluorescence anti-body (polyclonal goat anti-rabbit HRP) was purchased from KPL,

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Inc., (Gaithersburg, MD, USA). The sansalvamide analogue LY-15 was developed in Hebei Province Key Laboratory of Molecular Chemistry for Drug (Shijiazhuang, China).

Cancer cell line and cell culture. B16 cancer cells were selected for the present study. The B16 cell line was obtained from the Research Center of the Fourth Hospital of Hebei Medical University (Shijiazhuang, China). The cells were grown in RPMI 1640 medium with 10% heat-inactivated FBS and 100 µg/ml penicillin and streptomycin. The cell line was grown in a humidified atmosphere of 95% O₂ and 5% CO₂ at 37°C and the cells were periodically seeded into 25 cm² flasks. The media was changed every second or third day. For the experiments, the cells were grown to 80-90% confluence, digested with trypsin-EDTA, and plated in 25 cm² flasks, and the media was changed every second or third day on 6- or 96-well plates.

Concentration-dependent effect of LY-15 on B16 cell growth inhibition. LY-15 was dissolved in DMSO and diluted with serum-free medium to prepare solutions of 1,000, 100, 10 and 1 µM. Single-cell suspensions of B16 cells were prepared and adjusted to the indicated concentrations. The cells were inoculated in 96-well plates (100 µl/well) with 5,000 cells/well. After overnight inoculation for cell adherence, the old medium was discarded and replaced with fresh medium with different concentrations of 100, 50, 25, 15, 10, 5 and 1 µM. Each group was placed into six wells, and a 1% DMSO group was simultaneously prepared as the control. The CCK-8 method was used to calculate the percentage growth of the B16 cells treated with various concentrations of LY-15 for 24 h.

Time-dependent effect of LY-15 on B16 cell growth inhibition. Upon reaching 80% confluence, the cells were digested with trypsin-EDTA and serum-free medium was used to make a single-cell suspension. The cells were seeded over night in 96-well plates at a concentration of 3,000 cells/well. The wells were then replaced with fresh complete medium and treated with 10 µM LY-15. The percentage growth of the B16 cells treated for 24, 48, 72, 96 and 120 h was calculated.

Cell scratch test. Five uniform lines were drawn behind the 6-well plates using a marker pen. The single-cell suspension was seeded in the 6-well plates at a concentration of 200,000 cells/well. After 6 h, 20 µl pipette tips were used to draw through the marker lines. The wells were washed with PBS thrice and fresh media (2 ml/well) with different concentrations at 1, 2, 5 and 15 µM were added; moreover, 1% DMSO was added to the last well that was simultaneously prepared as the control. Images were captured at 0 and 24 h in the same position. We examined the effect of LY-15 on B16 cell migration using the cell scratch test.

Flow cytometric analysis of apoptotic cell death. At 80-90% confluence, the cells were treated with 2, 5, 10, 15 and 25 µM LY-15 for 24 h, and a control group was prepared. The treated and untreated cells were harvested, washed twice with PBS, and mixed with 1xbuffer 100 µl. After blending the cells, 10 µl FITC and 5 µl propidium iodide (PI) were added. The cells were kept in a dark place for 30 min.

Detection of caspase-3 and caspase-9 expressions using western blot analysis. The cells at 70-80% confluence were treated with 5, 15 and 25 µM of LY-15 for 24 h, and a control group was prepared. Proteins were separated using SDS-polyacrylamide gel electrophoresis. Equal amounts of protein (50 µg/sample) from B16 cells treated with LY-15 were loaded to 10% SDS-PAGE in an electrophoresis buffer in a Bio-Rad slab gel apparatus. The proteins were then transferred to a PVDF membrane under the conditions of 80 V for 20 min, 100 V for 90 min and 200 mA for 120 min. Next, the membranes were incubated in antibody dilution solution (rabbit anti-mouse bax, bcl-2, caspase-3 and caspase-9; 1:1,000 and β-actin; 1:3,000) overnight at 4°C. The blots were then incubated with the secondary antibody (1:3,000) for 1 h at 37°C. Results were obtained using the Aplegen Omega Lum C Gel Imaging System (Gel Company, Inc., San Francisco, CA). Concentration of protein was calculated by Image J (NIH, USA).

Statistical analysis. Statistical analysis was performed using SAS software (SAS Institute, Inc., Cary, NC, USA) and R programming language. Values were expressed as the mean ± standard error and were analyzed using one-way ANOVA followed by Tukey's post hoc test. P<0.05 was considered to indicate a statistically significant difference. All statistical analyses were conducted using Graphpad Prism v5 (GraphPad Software, Inc., La Jolla, CA, USA).

Results

LY-15 exhibits a concentration-dependent effect on B16 cell growth. The apoptosis and death of B16 cells were induced by the LY-15 treatment in various concentrations of (1, 5, 10, 15, 25, 50 and 100 µM) for 24 h. Moreover, the proliferation rate of the B16 cells showed a decreasing trend. Compared with the control group, no significant difference was identified for the proliferation rate of the 1% DMSO group (P>0.05), whereas those for the treatment of the B16 cells with 100, 50, 25, 15 and 10 µM LY-15 significantly decreased (P<0.001 for all treatment groups, Fig. 2). The morphological changes in B16 cells were observed via optical microscopy (Fig. 3). The B16 cells treated with 15 µM LY-15 for 24 h exhibited morphological changes, including decreased cell density, separation of the adjacent cells, and cell shrinkage.

LY-15 exhibits a time-dependent effect on B16 cell growth. The proliferation rate of the B16 cells with the treatment of the concentration of 100, 50, 25, 15, 10, 5 and 1 µM LY-15 was investigated. The concentration 10 µM LY-15 was significantly reduced in a time-dependent manner compared with that of the control group (P<0.001 for all time-dependent groups, Fig. 4). The results showed that LY-15 time-dependently inhibits the growth of B16 cells.

LY-15 inhibits the cell migration of B16 cells. Compared with the control group, no obvious difference was identified in the cell migration of the 1% DMSO group. The cell migration of B16 was weakened by the LY-15 treatments with concentrations of 1, 2, 5 and 15 µM for 24 h. The ability of cell migration gradually decreased as the concentration increased. The cell migration changes were observed under optical

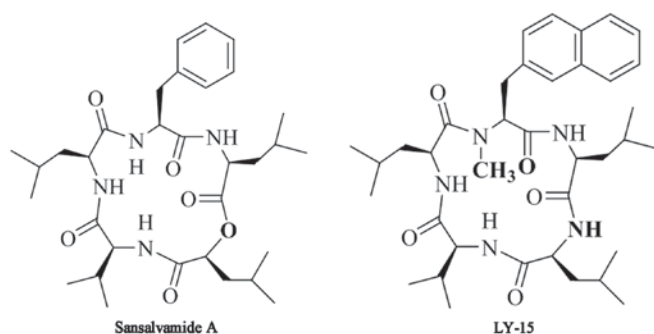


Figure 1. Structures of sansalvamide A and its analogue, LY-15.

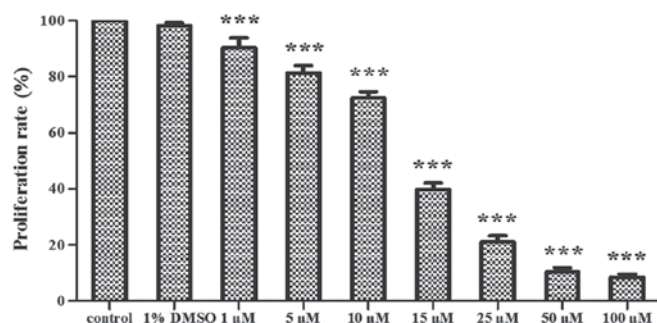


Figure 2. Compared with the control group, no significant difference was identified in the proliferation rate of the 1% DMSO group ($P>0.05$). All administration groups present significant differences compared with the control group ($P<0.001$). Besides, there was no significant difference between 50 and 100 μM ($P>0.05$). The overall average is not exactly equal ($F=943.214$, $P<0.001$). *** $P<0.001$.

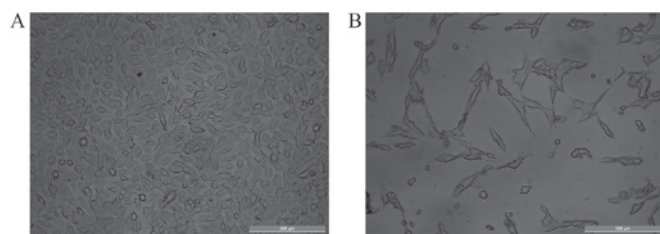


Figure 3. (A) B16 cells of the control group exhibiting vigorous and homogeneous growth; magnification, $\times 100$. (B) B16 cells treated with 15 μM LY-15 for 24 h exhibiting marked morphological changes, including decreased cell density, separation of the adjacent cells, and rounding of the cells; magnification, $\times 100$.

microscopy (Fig. 5). The cells lost their ability to migrate at LY-15 concentration of 15 μM .

LY-15 promotes the apoptosis of B16 cells. The ability of LY-15 to induce apoptosis was revealed by analyzing the cell samples using flow cytometry. The cells in the early stage of apoptosis were detected by Annexin V, whereas those in the late apoptosis were assessed by PI staining. The 1% DMSO group did not show significant difference in apoptosis compared with the control group. The early and late apoptosis stages of the B16 cells gradually increased with the treatment of LY-15 (5, 15 and 25 μM) for 24 h (Fig. 6). The percentage of apoptotic cells in the control group was 1.46; however, the proportion of apoptotic cells reached 8.57 in the group treated with 15 μM LY-15 for 24 h.

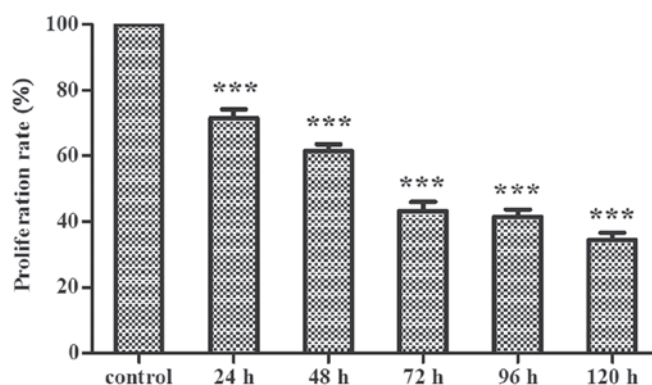


Figure 4. The B16 cells treated with 10 μM LY-15 showed significant differences in various time points compared with the control group ($P<0.001$). Besides, there was no significant difference between 72 and 96 h ($P>0.05$). The overall average is not exactly equal ($F=382.334$, $P<0.001$). *** $P<0.001$.

LY-15 induces apoptosis of B16 cells through the mitochondrial pathway. At the present stage of the study, the results of the western blot analysis showed an increasing trend in the expression of Bax, whereas that of Bcl-2 showed the opposite. Caspase-3 and caspase-9 expressions were analyzed using western blot analysis and further confirmed that LY-15 induces apoptosis. The expression levels of caspase-3 and caspase-9 in the B16 cells increased with the treatments of 5, 15 and 25 μM LY-15 (Fig. 7). All results revealed that LY-15 induces the B16 cells apoptosis through a mitochondrial pathway.

Discussion

Melanoma remains one of the most common cancers in western countries and is the main contributor to skin cancer-related deaths (10,11). Melanoma has a strong resistance and high metastasis and mortality rates (12,13). Studies were conducted on the synthesis and bioactivities of various sansalvamide A derivatives (14,15). The compound LY-15 is a novel sansalvamide A analogue that was recently synthesized by our group. Based on the specific structures of cyclic peptides, their bioactivities differ when the amino acid sequences are changed; moreover, the analogues are lipophilic and exhibit rapid membrane absorption (7). Accordingly, we studied the effects of the compound LY-15 on melanoma cell B16 and its potential molecular mechanisms to provide references for its clinical applications in melanoma therapy.

Melanoma cells exhibit strong proliferation, viability and malignancy. The curative effects of chemotherapeutic drugs against melanoma are clinically challenged because of their ability to resist apoptosis. Therefore, melanoma treatment studies have focused on finding and selecting a novel effective compound. Studies at this stage reported that the growth rate of B16 cells is significantly inhibited by the compound LY-15. Data showed that the effect of the compound LY-15 on the growth of B16 cells is concentration-(100, 50, 25, 15, 10, 5 and 1 μM) and time-dependent. Moreover, the cell proliferation rate was only 39.74% in the B16 cells cultured with 15 μM LY-15 for 24 h, indicating that the treatment has a remarkable effect. The effect of LY-15 on non-cancerous cells has been investigated preliminarily. The proliferation of 293t cell still kept more than 85% even as LY-15 with the concentration

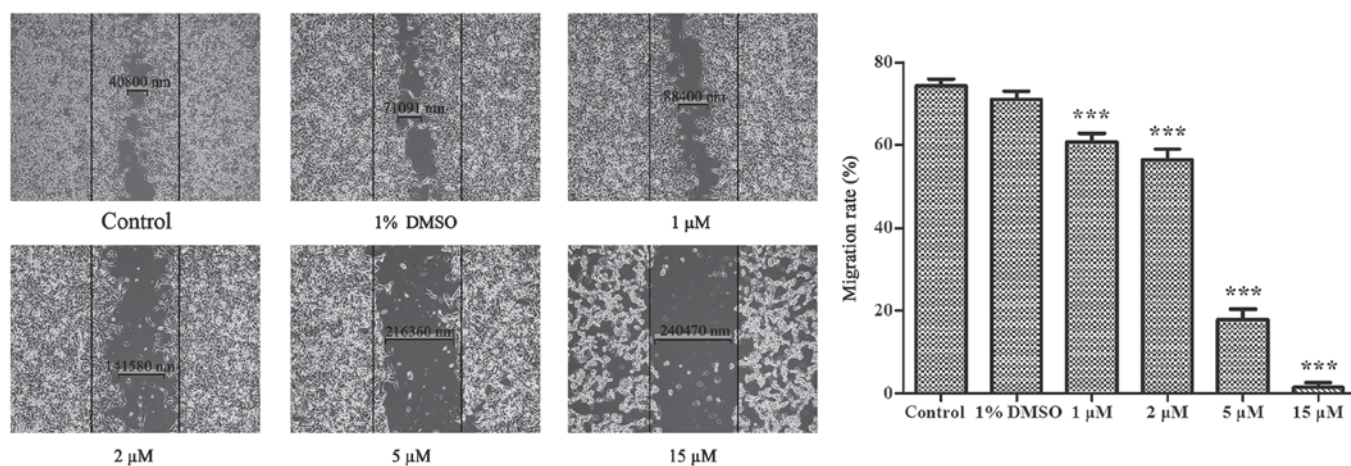


Figure 5. No significant difference was identified in the migration rate of the 1% DMSO group compared with that of the control group ($P>0.05$). LY-15 inhibited the cell migration of B16 cells. All administration groups present significant differences compared with the control group ($P<0.001$). Besides, there was no significant difference between 1 and 2 μ M ($P>0.05$). The overall average is not exactly equal ($F=622.366$, $P<0.001$). *** $P<0.001$.

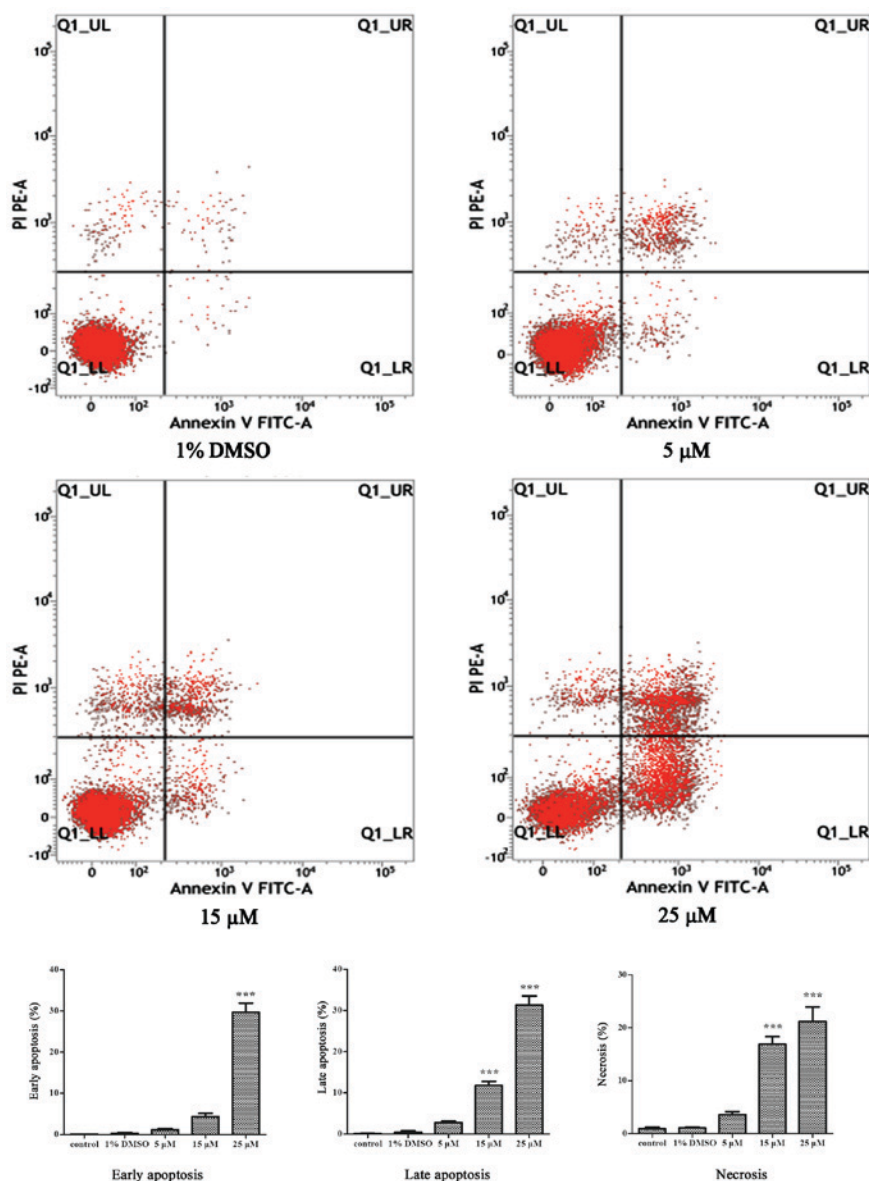


Figure 6. No significant difference was identified in the apoptosis rate of the 1% DMSO and 5 μ M groups compared with the control group ($P>0.05$). In the stage of early apoptosis, 25 μ M group present significant differences compared with the control group ($P<0.001$). In the stage of late apoptosis and necrosis, 15 and 25 μ M groups present significant differences compared with the control group ($P<0.001$). The overall average is not exactly equal ($P<0.001$). *** $P<0.001$.

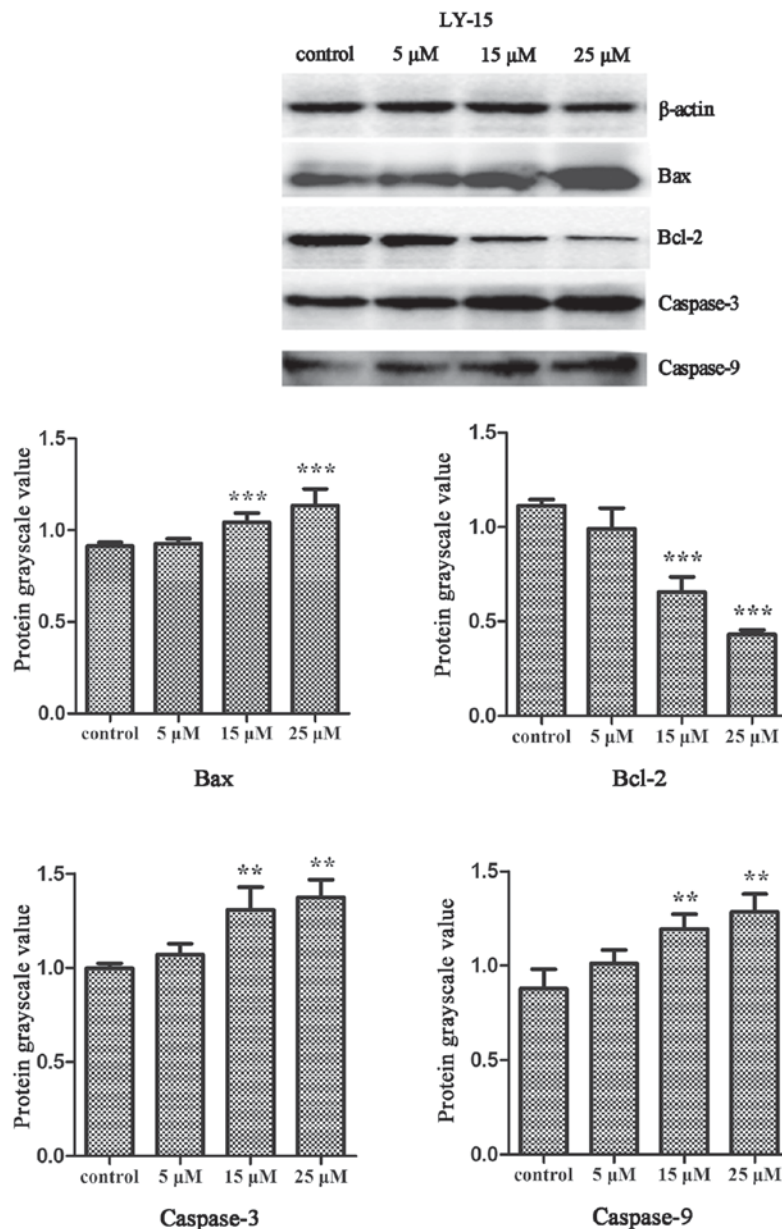


Figure 7. No significant difference was identified in the protein grayscale value of the 5 μM group compared with that of the control group ($P>0.05$). Besides, there was no significant difference between 15 and 25 μM ($P>0.05$). Compared with the control group, 15 and 25 μM groups present significant differences. For Bax and Bcl-2, $P<0.001$. For caspase-3 and caspase-9, $P<0.01$. The overall average is not exactly equal ($P<0.001$). ** $P<0.01$; *** $P<0.001$.

50 μM treated the cell. But, the details and the toxicities for more non-cancerous cell need to investigate further.

The cellular morphology of B16 treated with 15 μM LY-15 showed that the cell density was sharply reduced over time, and the cell exhibited a collection and spindle interstitial substance morphology. Moreover, B16 cells were significantly and slightly differentiated. Based on the flow cytometry data for B16 cells treated with 5 μM LY-15 for 24 h, cell apoptosis is significant, and the values of the early and late apoptotic stages of the B16 cells changed in a dose-dependent manner. These results revealed that the inhibition of B16 proliferation by the compound LY-15 is directly related to cell differentiation and apoptosis. Scratch wound healing assay was conducted to study the effect of LY-15 on the migration of B16 cells. The results showed that B16 cell migration was significantly inhibited by the 5 μM LY-15. In order to describe the details of tumor cell

movement, the cell invasion and cell cycle will be key work for further investigation. After the 15 μM LY-15 treatment, the cells turned sparse and apoptotic.

Apoptosis is programmed cell death and is an important part of the normal cell development and function of organisms. This process is triggered in a cell either through an extrinsic or intrinsic pathway (16). Protein Bcl-2 inhibits apoptosis in various cell types (17) and Bax is a protein that promotes cell apoptosis. Caspases play critical roles in apoptosis initiation and execution. Caspase-9, which is from the caspase family, is an initiator protein that drives caspase-3 to execute cell apoptosis. In investigating the anti-apoptotic potential of LY-15, the LY-15 administration significantly elevated the levels of the apoptotic marker proteins Bax, caspase-3 and caspase-9; whereas the anti-apoptotic factor Bcl-2 level was reduced following the treatment with various LY-15 concentrations (5, 15 and 25 μM). These results support

the hypothesis that LY-15 may inhibit B16 cell growth via the mitochondrial pathway, which induces apoptosis.

In conclusion, the results of this study showed that the compound LY-15 induces apoptosis in B16 cells and effectively inhibits their migration. Considering the invasiveness and drug resistance of melanoma, LY-15 provides a promising route for improving melanoma treatments.

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