Novel cyclic pentapeptide H-15 induces differentiation and inhibits proliferation in murine melanoma B16 cells

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Abstract. Sansalvamide A is a cyclic depsipeptide that is isolated from a marine fungus of the *Fusarium* genus. Sansalvamide A exhibits significant antitumor ability. The molecular formula and molecular weight of the novel sansalvamide A derivative H-15 are $C_{29}H_{44}BrN_5O_6$ and 637.2475, respectively. In the present study, H-15 was found to inhibit the proliferation and induce the differentiation of murine melanoma B16 cells. A sulforhodamine B colorimetric assay was used to measure the inhibitory effects of 0.1, 1, 10, 50 and 100 μM H-15 on the B16 cells, and the results revealed that the inhibitory effects of H-15 exerted on the B16 cells occurred in a concentration-dependent manner. In addition, the growth curve model of the B16 cells treated with 50 μM H-15 revealed that the effect of H-15 was also time-dependent. The differentiation morphology of the B16 cells was observed subsequent to treating the cells with H-15. An optical microscope was used to observe the differentiation morphology of the cells. In addition, melanin secretion increased in the B16 cells treated with 50 μM H-15. The expression levels of tyrosinase (TYR) were assayed by western blot analysis, and it was found that the cells treated with 50 μM H-15 for 48 h exhibited increased expression of TYR. The results of the present study indicated that H-15 may induce the differentiation of B16 cells.

Introduction

Malignant melanoma is a cancer that exhibits an increasing incidence and mortality rate, in addition to possessing a high risk of metastasis (1). Malignant melanoma is not sensitive to radiotherapy or chemotherapy; therefore, clinical treatment is an issue associated with malignant melanoma (2). In addition, the therapeutic effect of traditional chemotherapy is not sufficient to treat this disease, and the side effects of the chemotherapy drugs may cause marked damage to the patient (2). Therefore, effective and low-toxicity compounds that treat malignant melanoma are required to be identified or developed. Previous studies have found that several tumor cells exhibit differentiation defects (3,4); however, treating tumor cells with compounds may cause normal differentiation and reduce tumor malignancy, for example, all-trans retinoic acid (atRA) is used in the differentiation therapy of acute promyelocytic leukemia (4). Sansalvamide A, which is a cyclic depsipeptide derived from a marine fungus of the *Fusarium* genus, exhibits significant antiproliferative effects in the 60 cancer cell line panel of the National Cancer Institute (5). Synthesis of sansalvamide A derivatives has received increasing attention and novel sansalvamide A derivatives may be valuable therapeutic agents (6-8). The effect of the novel sansalvamide A derivative and a cyclic pentapeptide H-15 on the growth and differentiation of murine malignant melanoma B16 cells was investigated in the present study. H-15 possesses a molecular formula and molecular weight of $C_{29}H_{44}BrN_5O_6$ and 637.2475, respectively (Fig. 1). In the present study, the results may provide a basis for additional studies of this novel compound.

Materials and methods

Materials. Gibco RPMI-1640 and trypsin-ethylenediaminetetraacetic acid (EDTA) solution were purchased from Sigma-Aldrich (St. Louis, MO, USA). Fetal bovine serum (FBS) was purchased from Hangzhou Sijiqing Biological Engineering Materials Co., Ltd. (Zhejiang, China). Dimethyl sulfoxide (DMSO) was purchased from Tianjin Yongda Chemical Reagents Development Center (Tianjin, China) H-15 was provided by the Hebei Province Key Laboratory of Molecular Chemistry for Drug (Shijiazhuang, Hebei, China). Sulforhodamine B (SRB) was purchased from Tokyo Chemical Industry Co., Ltd. (Tokyo, Japan) and bicinchoninic acid (BCA) kit was acquired from Shanghai Generay Biotechnology Co., Ltd. (Shanghai, China). Polyvinylidene

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fluoride (PVDF) membranes were purchased from Shanghai Generay Biotechnology Co., Ltd., and the polyclonal rabbit anti-mouse glyceraldehyde-3-phosphate dehydrogenase (GAPDH; cat no. 2118s) antibody was obtained from Hangzhou Goodhere Biotechnology Co., Ltd. (Hangzhou, Zhejiang, China). The monoclonal rabbit anti-mouse tyrosinase (TYR; cat no. sc15341) antibody was purchased from Santa Cruz Biotechnology, Inc. (Dallas, TX, USA), and the secondary polyclonal goat anti-rabbit fluorescence-conjugated antibody was purchased from LI-COR Biosciences, Ltd. (Cambridge, UK; cat no. 926-32211). The B16 cell line was stored at the Research Center of the Fourth Hospital of Hebei Medical University (Shijiazhuang, Hebei, China).

Cell culture. The B16 cells were cultured in RPMI-1640 medium, together with 10% heat-inactivated FBS and 100 U/ml penicillin and 100 μg/ml streptomycin. The B16 cell line was grown in 25 cm^2 flasks in a humidified atmosphere of 5% CO_2 at 37˚C, and the media were changed every 2-3 days. The B16 cells were allowed to grow to 80-90% confluency, and the cells were then digested using trypsin-EDTA. The cells were subsequently plated in 25 cm^2 flasks and in 24- or 96-well plates for generation of the cells and use in additional experiments.

Concentration-dependent effect of H-15 on B16 cell growth inhibition. H-15 was dissolved in dimethyl sulfoxide (DMSO) and diluted with a serum-free medium to prepare solution concentrations of 1,000, 500, 100, 10 and 1 μM. Single-cell suspensions of B16 cells were prepared and adjusted based on the indicated concentrations. The cells were then inoculated in 96-well plates, with 90 μl of cell solution and ~2,000 cells/well. The cells were allowed to adhere to the plates for 4 h, and 10 μl H-15 was added to each well to produce the final concentrations of 100, 50, 10, 1 and 0.1 μM H-15. Each concentration was placed in three wells, and a 1% DMSO group was simultaneously prepared as the control group. The percentage growth of the B16 cells treated with various concentrations of the H-15 for 48 h was calculated using the SRB colorimetric method.

SRB colorimetric method. The cells were fixed with trichloroacetic acid (TCA) following treatment with H-15 for 48 h, and the intracellular protein was stained with SRB. A total of 100 μl of Trisbase was then added to each well. The dissolved SRB was detected using a microplate reader (Thermo Fisher Scientific, Inc.), wherein the values indirectly indicated the numbers of living cells. The medium in the 96-well plates was discarded, and 100 μl TCA was added at a temperature of 4˚C for 30 min. The TCA was then discarded, and the cells were washed three times (30 sec washes) with distilled water prior to drying at room temperature for 1 h. A total of 100 μl of 0.4% SRB was then added to the cells, and the cells were agitated for 20 min. The dye solution was discarded, and the cells were washed three times with 1% acetic acid and dried at room temperature for >6 h. Finally, 100 μl Trisbase was added, and the cells were again agitated for 5 min. The optical density (OD) was obtained at a wavelength of 490 nm using a Multiskan Go microplate reader (Thermo Fisher Scientific, Inc.).

Time-dependent effect of H-15 on B16 cell growth inhibition. The cells were harvested at 80-90% confluency using trypsin, and a serum-free medium (RPMI-1640 medium without FBS) were used to produce a single-cell suspension (10,000 cells/ml). The cells were seeded in 24-well plates at a concentration of 20,000 cells/well. The medium and FBS in the wells was replaced with fresh medium and FBS subsequent to 24 h. The wells were then treated with 50 μM H-15, and the cell numbers were counted following 24, 48, 72, 96, 120 and 144 h of treatment. A control group (with equal volume of serum-free RPMI-1640 medium) was simultaneously prepared, and a growth curve was generated.

Detection of melanin content of B16 cells. The cells were harvested at 80-90% confluency using trypsin, and a serum-free medium was used to produce a single-cell suspension. The cells were then seeded in 25 cm^2 flasks with ~50,000 cells/flask. The cells were allowed to adhere in the flask for 4 h, and H-15 was added to the test flasks. The final concentration in the flask was then adjusted to 50 μM. The cells were treated for 48 h and harvested using trypsin. The cell pellets were collected and washed twice with 0.9% NaCl. The pellets were then dissolved in 200 μl of 1 M NaOH solution containing 10% DMSO, and were then placed in a water bath at 80˚C for 2 h. The pellets were agitated for 30 sec to dissolve the melanin and centrifuged at 300 x g for 5 min, to remove the precipitates. The liquid was transferred onto 96-well plates, and the optical density was obtained at a wavelength of 490 nm using a Multiskan Go microplate reader. Cell viability was calculated as follows: Viability (%)=[experimental group (OD) - blank group (OD)]/[control group (OD) - blank group (OD)] X 100.

Detection of TYR expression by western blot analysis. Once the cells reached 80-90% confluency, they were treated with 50 μM H-15 for 24 h, and a control group (without H-15) was prepared. The cellular protein was extracted using a radioimmunoprecipitation assay lysis buffer (BestBio company, Shanghai, China), and the concentration of the extracted protein was measured using a BCA kit. A total of 50 μg protein was electrophoretically separated on a 10% polyacrylamide gel. The proteins were transferred to a PVDF membrane with 90 V and 200 mA for 60 min. The membranes were then incubated with rabbit anti-mouse TYR and GAPDH antibodies (dilution, 1:500) overnight at 4˚C. The blots were then incubated with the secondary fluorescence-conjugated antibody (dilution, 1:5,000) for 2 h in the dark, and the results were obtained using an Odyssey infrared imager (LI-COR, Inc., Lincoln, NE, USA).

Statistical analysis. Statistical analysis was performed using SPSS software, version 13.0 (SPSS, Inc., Chicago, IL, USA). The data were presented as the mean ± standard error of the mean and were analyzed by paired *t*-test. $P<0.05$ was considered to indicate a statistically significant difference.

Results

H-15 exerts a concentration-dependent effect on B16 cell growth. No significant difference was observed between in the proliferation rate of the 1% DMSO and the control

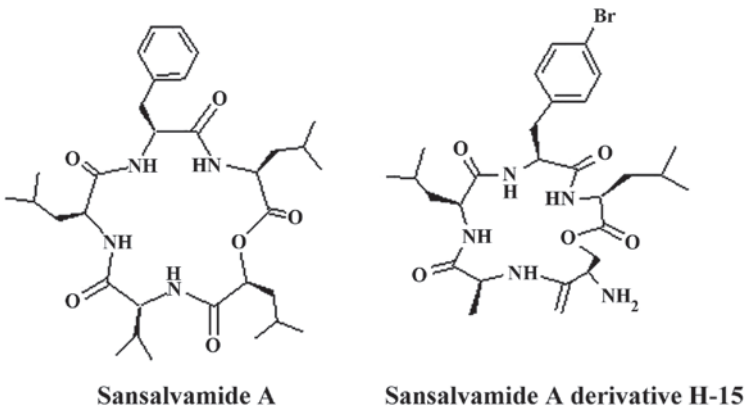

Figure 1. Structure of sansalvamide A and its derivative, H-15.

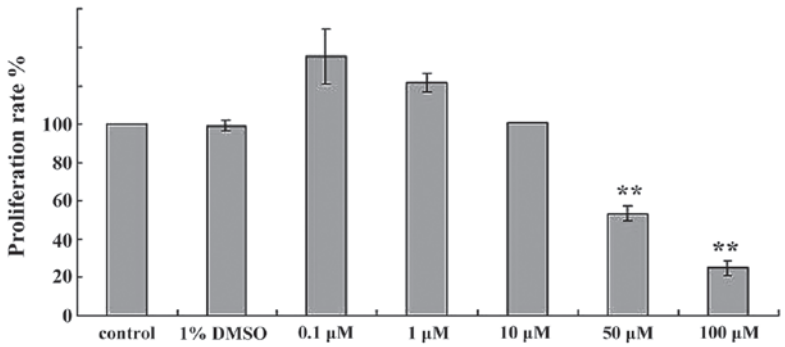

Figure 2. Effect of H-15 on the proliferation of B16 cells measured using the sulforhodamine B colorimetric method. Compared with the control group, no significant difference was identified in the proliferation rate of the 1% DMSO group (P>0.05). However, H-15 was found to cause concentration-dependent inhibition of B16 cell proliferation. **P<0.01 vs. control. DMSO, dimethyl sulfoxide.

groups (P>0.05). The proliferation rate of the B16 cells gradually decreased subsequent to the treatment of the cells with increasing concentrations of H-15 (0.1, 1, 10, 50 and 100 μM) for 48 h compared with the proliferation rate of the control group cells. The proliferation rate of the B16 cells treated with 100 and 50 μM H-15 was also significantly decreased (100 μM, P<0.01; 50 μM, P<0.01; Fig. 2) compared with the proliferation rate of the control group. Morphological changes in the cell were observed under light microscopy (Fig. 3). The B16 cells treated with 50 μM H-15 for 48 h exhibited marked morphological changes, including decreased cell density, increased cell volume and more evident cell processes.

H-15 exhibits a time-dependent effect on the growth of B16 cells. The time-dependent effect of H-15 on cell proliferation was measured in terms of the cell number. The number of B16 cells was counted subsequent to treatment of the cells with 50 μM H-15 for 24, 48, 72, 96, 120 and 144 h. These cell numbers were then compared with those of the control group. The results indicated that H-15 inhibited the growth of the B16 cells in a time-dependent manner (Fig. 4).

H-15 caused B16 cells to increase production of melanin. The melanin content in the cells treated with 50 μM H-15 for 48 h was increased compared with the melanin content of the control group. The OD value of the B16 cells following treatment with 50 μM H-15 for 48 h was 0.1743±0.0227, whereas the OD of the control group was 0.0788±0.0039. The difference in the results was statistically significant (P<0.05; Fig. 5).

H-15 may increase the expression of TYR. TYR is an enzyme that plays an important role in melanin production. The cells treated with 50 μM H-15 for 48 h exhibited increased expression of TYR (Fig. 6). These results indicated that H-15 may induce the differentiation of B16 cells.

Discussion

Malignant melanoma is a cancer that exhibits an increasing incidence and mortality rate, a high metastasis rate and a strong resistance to chemotherapy and radiotherapy (1). Poor prognosis is evident in patients with malignant melanoma. Currently, effective methods of treatment, or drugs that treat malignant melanoma, are not available (1). Therefore, novel methods and drugs are necessary for the treatment of this disease. Sansalvamide A, which is a cyclic depsipeptide isolated from a marine fungus of the *Fusarium* genus, has exhibited marked anti-tumor effects in the 60 cancer cell line panel of the National Cancer Institute (5). Various sansalvamide A derivatives have been synthesized, and these sansalvamide A derivatives have demonstrated strong antitumor ability and good stability (9,10).

Malignant melanoma, similar to stem cells, shows an extremely strong proliferation capability. At present, induced

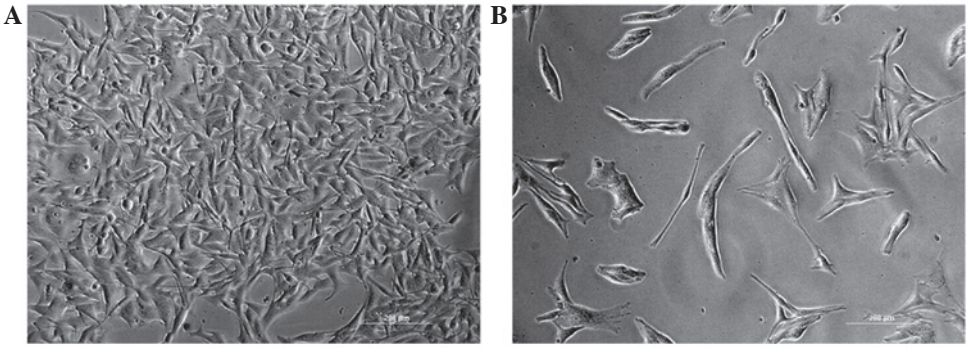

Figure 3. (A) B16 cells of the control group exhibiting vigorous and homogenous growth. Magnification, x100. (B) Subsequent to treatment with 50 μM H-15 for 48 h, B16 cells exhibited an increased cell volume and decreased density. Magnification, x100.

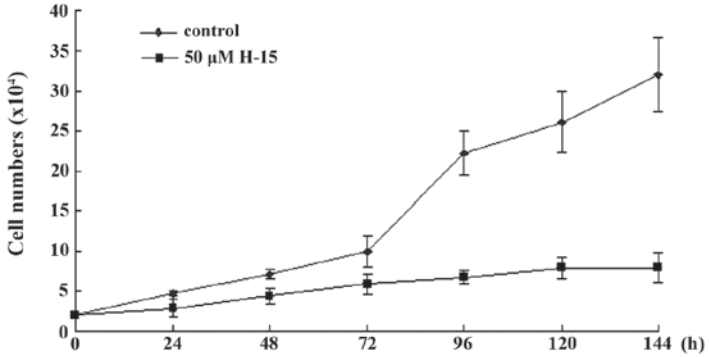

Figure 4. Time-dependent effect of 50 μM H-15 on B16 cell growth. The upper line in the graph represents the control group without H-15 treatment and the bottom line presents the group treated with H-15. At the end of each time-period, the cells were trypsinized to produce a single cell suspension and the cell number was counted. Data are present as the mean ± standard error of the mean.

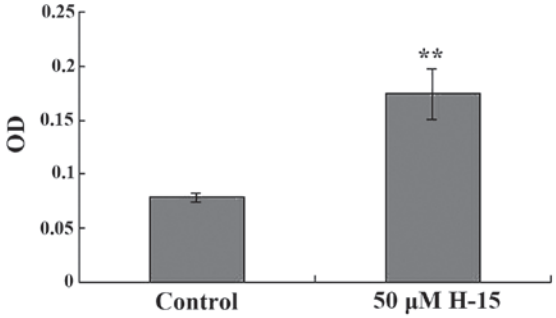

Figure 5. OD values of melanin produced by B16 subsequent to treatment with 50 μM H-15 for 48 h. **P<0.01 vs. control group. OD, optical density.

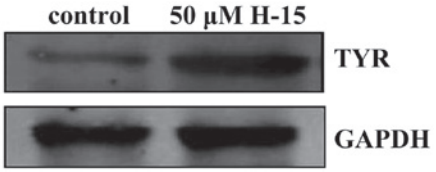

Figure 6. Western blot analysis of TYR expression. The results revealed an ascendant trend in the expression of TYR subsequent to treatment with 50 μm H-15. TYR, tyrosinase; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

differentiation therapy for cancer has received increasing attention (11). Inducing tumor cells to lose stem cell properties and enabling these cells to exhibit the specific functions of differentiated cells is the theoretical basis of induced differentiation therapy, such as inducing melanocytes to produce melanin. Normal or malignant cells exhibit a decreased proliferative ability following differentiation. The present study showed that H-15 significantly inhibited the proliferation rate of B16 cells, and the time-dependent analysis confirmed that H-15 demonstrated a long-lasting suppression effect on the growth of the B16 cell line.

At present, induction differentiation therapy for the treatment of cancer is receiving increasing attention (3,4). Inducing tumor cells to lose stem cell properties and enabling these cells to exhibit the specific functions of differentiated cells is the theoretical basis of induced differentiation therapy, and results in the cells losing the ability for proliferation and invasion (12). Melanin pigments are released by melanocytes, and the color of skin and hair are largely determined by melanin. Melanin is derived from the precursor dopaquinone that is formed by TYR oxidation of L-tyrosine, and therefore, TYR plays an important role in melanin synthesis (13). The ability to produce melanin and the upregulation of TYR were proposed to be responsible for the differentiation of B16 cells. In the present study, the melanin content of cells was evaluated and the results showed that the melanin level was higher in cells subsequent to treatment with 50 μM H-15 for 48 h compared with the control cells. TYR is a important component in the production of

melanin, and following treatment with 50 μM H-15 for 48 h, the results of the western blot analysis revealed an ascendant trend in the expression of TYR. These results indicated that H-15 may induce the differentiation of B16 cells.

In conclusion, the results of the present study indicated that H-15 may induce the differentiation of murine melanoma B16 cells. In addition, this novel compound may improve the therapeutic approach for the treatment of melanoma.

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