

Alternol inhibits the proliferation and induces the differentiation of the mouse melanoma B16F0 cell line

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Abstract. High malignant potential and low susceptibility to treatment are characteristics of malignant melanoma. Alternol, a novel compound purified from microbial fermentation products obtained from the bark of the yew tree, exhibits a variety of antitumor activities. Based on these findings, the aim of the present study was to extend the knowledge on the antineoplastic effect of alternol in the mouse melanoma B16F0 cell line. Alternol significantly inhibited the proliferation and colony formation of B16F0 cells in a dose-dependent manner as detected by MTT and soft agar colony formation assays. NaOH alkaline lysis and oxidation of Dopa indicated that alternol enhanced the melanin content and tyrosinase activity of the B16F0 cells and results also showed a dose-response relationship. Morphologic changes accompanied by extended dendrites were discovered in the B16F0 cells after treatment with alternol. Furthermore, the mRNA levels of tyrosinase, *Trp1* and *Trp2* were increased by alternol. Our results confirmed that alternol possesses marked antineoplastic properties against melanoma cells, indicating that this microbial fermentation product is a promising agent for the differentiation therapy of cancer. The inhibition of cell proliferation and colony formation by alternol was associated with both cytotoxicity and induction of differentiation.

Introduction

Melanoma is a malignant tumor which originates from melanocytes or cells evolved from melanocytes. Malignant melanoma is the most common cancer located on the back in men and on the legs in women. Each year in the world, the number of estimated new cases of malignant melanoma is 132,000 and approximately 48,000 patients die from malignant melanoma. Rapid growth, early and multiple metastases

and low susceptibility to treatment determine the significant malignant potential of melanoma. The prognosis for patients with advanced melanoma is grim, with a 1-year survival rate of 25% and a median overall survival of 6.2 months (1,2).

For advanced melanoma, systemic therapy is usually needed; however, therapeutic options for unresectable or metastatic melanoma are limited. Many patients are resistant to conventional chemotherapies with alkylating agents, dacarbazine or interferon (IFN)- α (3,4). In recent years, due to the low effectiveness of chemotherapy and radiotherapy in anti-melanoma treatment, alternatively, inducers of differentiation warrant therapeutic importance. Some studies have demonstrated that malignant cancer cells could be transformed into mature cells by induction of differentiation (5,6). Several compounds including dimethyl sulfoxide, retinoic acid, phorbol ester and 1,25-dihydroxy vitamin D3 are known to induce acute promyelocytic leukemia (AML) cells to differentiate toward mature cells (7,8). More intriguingly, induction of differentiation can enhance bortezomib efficacy and overcome drug resistance in multiple myeloma (9).

The B16F0 cell line, which was derived from C57BL/6 mice, provides a useful cellular differentiation model. The terminal differentiation of B16F0 cells can be monitored by changes in morphology, upregulation of melanin biosynthesis, and induction of dendrite outgrowths. The differentiation of melanoma cells into a terminal stage may be an effective strategy for the treatment of melanoma.

Alternol, a novel compound purified from microbial fermentation products obtained from the bark of the yew tree, exhibits a variety of antitumor activities, including proliferation inhibition, cell cycle arrest, apoptosis, and suppression of migration and invasion (10-15). However, the anticancer effect and molecular mechanisms of alternol have not yet been established in B16F0 cells. Here, we report the effects of alternol on the proliferation and differentiation potential of B16F0 cells.

Materials and methods

Materials and reagents. Alternol with 99.5% purity was acquired from Shantou Strand Biotech Co., Ltd. The chemical structure of alternol is shown in Fig. 1. The alternol stock solution at 10 mmol/l was made in dimethyl sulfoxide (DMSO) and stored in the dark at -20°C. Dulbecco's modified Eagle's

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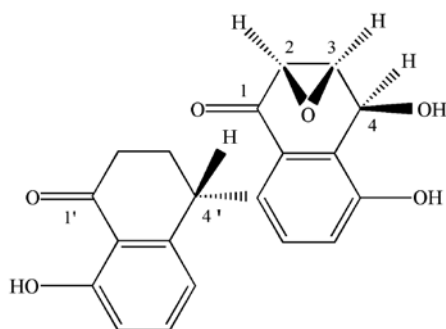


Figure 1. Chemical structure of alternol.

medium (DMEM) and fetal bovine serum (FBS) were purchased from Gibco Laboratories (Grand Island, NY, USA). L-DOPA, thiazolyl blue (MTT) and Triton X-100 were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Antibiotics such as penicillin and streptomycin were obtained from Shandong Lukang Pharmaceutical Co., Ltd. (Shandong, China). All other chemicals were of analytical grade and commercially available.

Cell culture. B16F0 cells were obtained from the China Center for Type Culture Collection (Wuhan, China). The cells were cultured in DMEM supplemented with 10% FBS, 100 U/ml penicillin and 100 $\mu\text{g/ml}$ streptomycin. The cells were maintained at 37°C in a humidified incubator with 5% CO₂ atmosphere. Logarithmically growing B16F0 cells were used for each experiment, and the density of inoculation was $\sim 1 \times 10^5$ cells/ml. In order to avoid changes in cell characteristics that are caused by prolonged cell culture time, the cells were used between passages 15 and 25.

Cell proliferation assay. For the cell proliferation assay, B16F0 cells were inoculated into 96-well plates at $\sim 5 \times 10^4$ cells/well. Subsequently, the cells were treated with alternol in a range of concentrations: 0, 0.4, 1.6, 2.4, 3.2, 4.0 $\mu\text{g/ml}$. The effect of alternol on the growth of B16F0 cells was assessed by MTT assay as previously described (16,17). The absorbance at 490 nm was measured using a microplate reader (Thermo Varioskan Flash 3001; Thermo Scientific).

Morphological changes of B16F0 cells. Logarithmically growing B16F0 cells were inoculated into 6-well plates at a concentration of 1×10^5 cells/well. After cells were grown on the glass slides, different concentrations of alternol 0–2.4 $\mu\text{g/ml}$ were added at 37°C for 24 h (18). Then the cells were observed using a phase-contrast microscopy (Zeiss).

Trypan blue exclusion test. The survival rate of B16F0 cells was assessed by the trypan blue exclusion test as previously described (19,20). Cells in the exponential growth phase were seeded in 6-well plates at 5×10^4 cells/well. After a 24-h growth period, the cells were treated with different concentrations of alternol at 37°C for 24 and 48 h. After being digested with trypsin, the viable and dead cells were collected for counting using an optical microscope with a hemacytometer. Trypan blue exclusion test was used to record the number of living cells for each group. Cell survival rate (%) = (total number of

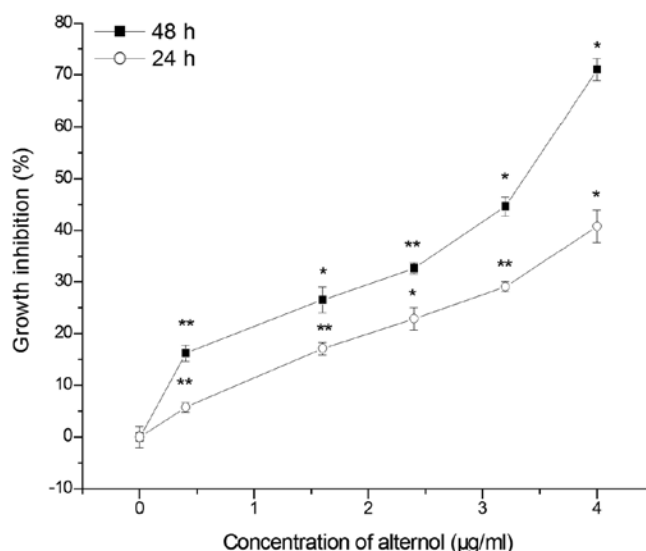


Figure 2. Effects of alternol on the proliferation of B16F0 cells. The inhibitory growth rate was determined by MTT assay after 24 or 48 h of incubation. Data are presented as means \pm SEM from six samples of three independent experiments. * $P < 0.05$, ** $P < 0.01$ vs. vehicle control by ANOVA followed by the Student-Newman-Keuls test.

viable cells per ml of aliquot)/(total number of cells per ml of aliquot) $\times 100\%$.

Soft agar colony formation assay. Anchorage-independent cell growth of B16F0 cells was assessed by the soft agar formation assay. Cells for each group were harvested by trypsinization, and then a single-cell suspension in DMEM was plated into 6-well plates containing 0.35% low melting agarose and solidified 0.6% agarose. Cells in agar were incubated at 37°C in a humidified environment for ~ 2 weeks. The colonies were counted directly and photographed by an imaging system as previously described (21,22).

Determination of melanin content. The melanin content was measured as described in previous studies with slight modifications (23,24). Melanoma B16F0 cells were seeded at a density of 1×10^5 cells/well in 6-well plates. After incubation for 24 h at 37°C, the cells were treated with different concentrations of alternol for 48 h. The supernatant was collected separately. Extracellular melanin content was measured as previously described. Upon the determination of intracellular melanin content, adhered cells were washed with PBS and digested with 0.25% trypsin. The cells were then centrifuged at 12,000 rpm for 10 min. Then the mixture consisting of 0.4 M HEPES buffer (pH 6.8) and EtOH (9:1, v/v) was added to the cells. Melanin was dissolved in the mixture after incubation at 42°C for 16 h. Then the solution was transferred into 96-well plates and observed at 475 nm using a microplate reader.

Cellular tyrosinase activity assay. For the determination of tyrosinase activity, we used a previously reported method (25,26). According to the description of Dopa oxidation method, B16F0 cells were seeded in 6-well plates at a concentration of 1×10^5 cells/well. The cells were collected and washed with ice-cold PBS prior to centrifugation. After incubation at

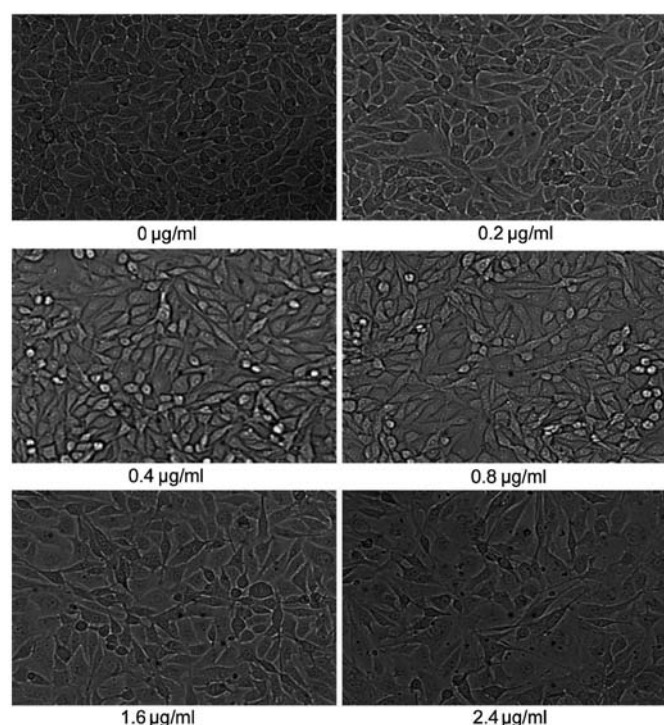


Figure 3. Morphological changes in B16F0 cells after alternol treatment. Cells were treated with 0, 0.2, 0.4, 0.8, 1.6 and 2.4 $\mu\text{g/ml}$ alternol for 24 h, and photomicrographs were captured using a digital video camera. Micrographs were collected on a Zeiss microscope at x200 magnification.

-80°C for 30 min, the cells were lysed with buffer containing 1% Triton X-100 and PMSF (0.1 mM). The cell lysate was thawed, mixed and centrifuged at 12,000 rpm for 30 min to obtain the supernatant. The mixture of 80 μl of supernatant and 20 μl of L-DOPA were placed in a 96-well plate and incubated at 37°C for 40 min. The absorbance was measured at 475 nm.

Reverse transcription-polymerase chain reaction (RT-PCR). B16F0 cells were pretreated with alternol (0, 0.4, 0.8, 1.6, 2.4, 3.2 $\mu\text{g/ml}$) as protocols planned in advance. Total RNA was isolated using TRIzol reagent as previously described (27). Reverse transcription of cDNA was accomplished using a cDNA synthesis kit (Promega, Madison, WI, USA). cDNA was synthesized in a 25- μl reaction system by adding 3 μl total RNA primed with oligo(dT) (deoxy-thymidine). The sequences of the primers were as follows: tyrosinase upstream, 5'-GGC CAG CTT TCA GGC AGA GGT-3' and downstream, 5'-TGG TGC TTC ATG GGC AAA ATC-3'; TRP-1 upstream, 5'-GCTGCA GGA GCC TTC TTT CTC-3' and downstream, 5'-AAG ACG CTG CAC TGC TGG TCT-3'; and TRP-2 upstream, 5'-GGA TGA CCG TGA GCA ATG GCC-3' and downstream, 5'-CGG TTG TGA CCA ATG GGT GCC-3'. The level of GAPDH (upstream, 5'-CAA GGT CAT CCA TGA CAA CTT TG-3' and downstream, 5'-GTC CAC CAC CCT GTT GCT GTA G-3') was added as a control. PCR was performed in a mixture containing cDNA, 10X PCR buffer, 2.5 mM dNTPs, 10 mM forward and reverse primers, DNA polymerase and sterile water.

Statistical analysis. All data are presented as the mean \pm SE from at least three independent experiments and were

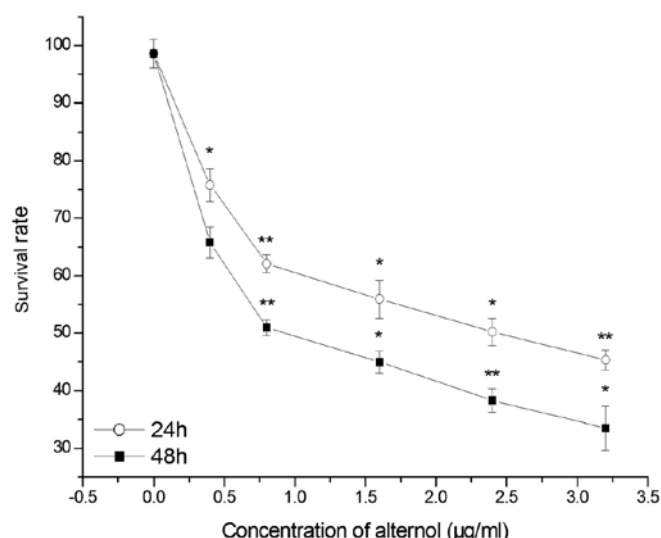


Figure 4. Alternol reduces cell survival rate. The survival rate of the B16F0 cells was detected by trypan blue exclusion test after alternol treatment for 24 and 48 h. Data are presented as means \pm SEM from six samples of three independent experiments. * $P < 0.05$, ** $P < 0.01$ vs. vehicle control by ANOVA followed by the Student-Newman-Keuls test.

evaluated by analysis of variance (ANOVA) followed by the Student's t-test. Values of $P < 0.05$ were considered to indicate statistically significant results.

Results

Alternol inhibits the proliferation of B16F0 cells. The anti-tumor effect of alternol on B16F0 cells was monitored by MTT method. As shown in Fig. 2, alternol significantly inhibited the proliferation of B16F0 cells. When cells were exposed to alternol at 4 $\mu\text{g/ml}$ for 48 and 24 h, the inhibitory growth rate of alternol in the B16F0 cells was 71.03 ± 2.17 and $40.74 \pm 3.16\%$, respectively. Moreover, the cell proliferation of the B16F0 cells was reduced by alternol in a dose-and time-dependent manner.

Morphological changes in the B16F0 cells after alternol treatment. Morphological changes in the B16F0 cells were observed after treatment with different concentrations of alternol (0, 0.2, 0.4, 0.8, 1.6 and 2.4 $\mu\text{g/ml}$) for 24 h (Fig. 3). With the increase in alternol concentration, cells exhibited dendritic-like projections which gave a star-like shape to the cells compared with the rounded untreated cells. These branches in cells become increasingly evident. The exposure of B16F0 cells to alternol resulted in a marked differentiation phenomenon.

Alternol reduces the cell survival rate. The dye exclusion test is based on the principle that live cells possess intact cell membranes that exclude certain dyes, such as trypan blue, eosin and propidium, whereas dead cells do not. This method was used to determine the number of viable cells present in the cell suspension. In this experiment, we used trypan blue as a target dye. The results showed that after 24 and 48 h of exposure to alternol (0-2.4 $\mu\text{g/ml}$), the survival rate of the B16F0 cells was decreased in a dose-dependent manner (Fig. 4). The survival rate of the B16F0 cells was only 46% after 1.6 $\mu\text{g/ml}$ alternol

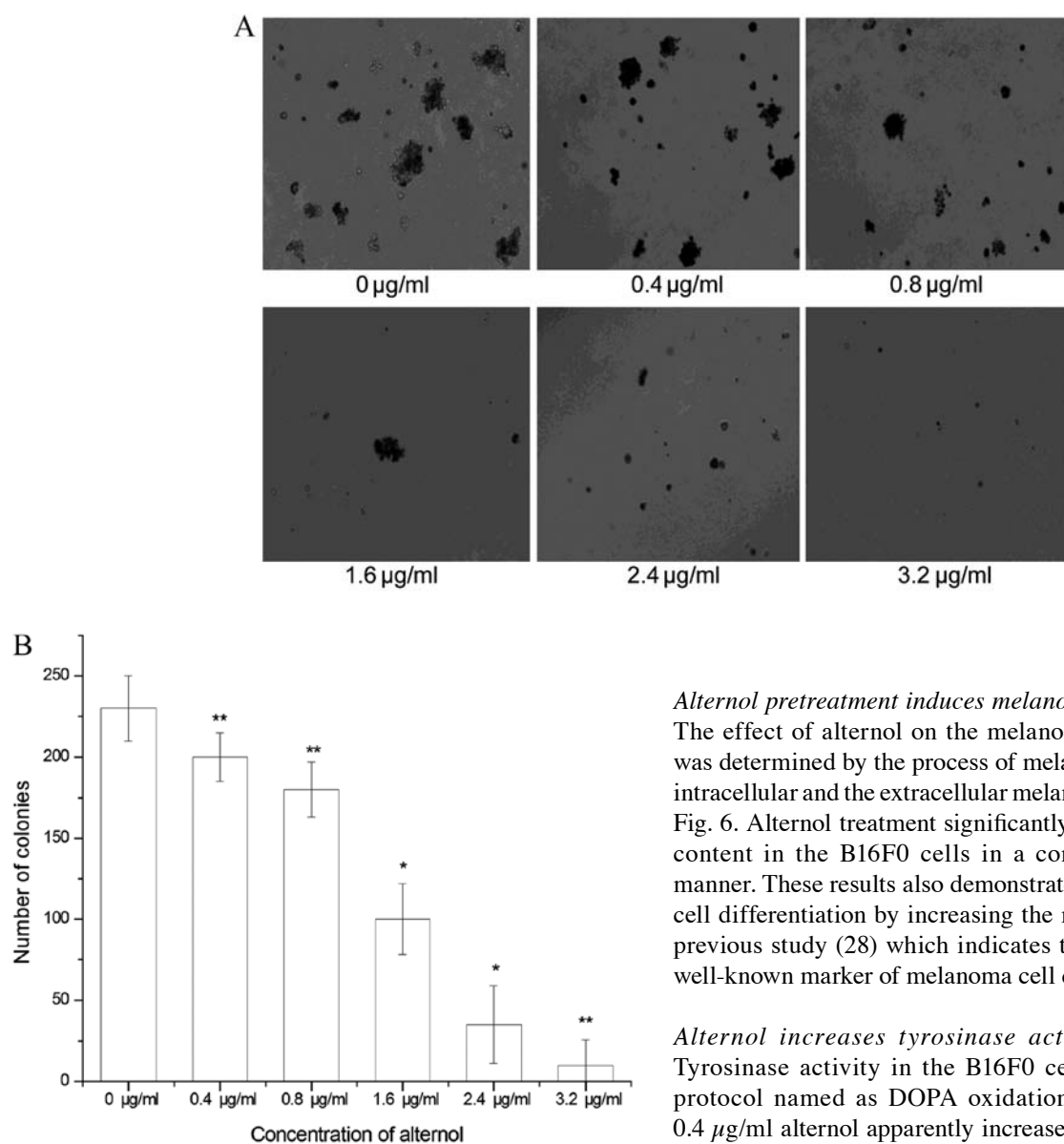


Figure 5. Alternol inhibits colony formation in the B16F0 cells. The anchorage-independent growth of B16F0 cells treated with alternol were examined by soft agar colony formation assay. (A) Colonies were photographed and representative phase contrast images are shown. (B) Colonies were counted under a microscope. Data are presented as the mean \pm SEM for three independent experiments. * $P < 0.05$, ** $P < 0.01$ as compared to the control.

stimulation. Together these results confirmed that alternol significantly affected cell survival even at low concentrations (0.2–0.8 µg/ml).

Alternol inhibits the colony formation of B16F0 cells. Colony-forming ability in soft agar provides strong evidence for the tendency of tumor cells to undergo neoplastic transformation. The colony-forming efficiency of the B16F0 melanoma cells is exhibited in Fig. 5A. The numbers of colonies observed within a field of vision under a microscope are shown in Fig. 5B. Results revealed that alternol inhibited both the number and the size of colonies, which indicates that alternol can effectively suppress tumorigenicity *in vitro*.

Alternol pretreatment induces melanogenesis of B16F0 cells. The effect of alternol on the melanogenesis of B16F0 cells was determined by the process of melanin synthesis. Both the intracellular and the extracellular melanin content are shown in Fig. 6. Alternol treatment significantly increased the melanin content in the B16F0 cells in a concentration-dependent manner. These results also demonstrated that alternol induced cell differentiation by increasing the melanin content as in a previous study (28) which indicates that melanogenesis is a well-known marker of melanoma cell differentiation.

Alternol increases tyrosinase activity in B16F0 cells. Tyrosinase activity in the B16F0 cells was assessed by a protocol named as DOPA oxidation. As shown in Fig. 7, 0.4 µg/ml alternol apparently increased the activity of tyrosinase in the alternol-treated cells compared with the control group. Alternol treatment also increased tyrosinase activity in a dose-dependent manner, which was consistent with the increase in melanin content in the alternol-treated cells.

Alternol increases the mRNA level of genes related to melanogenesis. There are many complex networks in the process of melanin synthesis. It is well known that tyrosinase, TRP-1 and TRP-2 are all key factors during the process of melanogenesis. As shown in Fig. 8, alternol significantly enhanced the mRNA levels of tyrosinase, *TRP-1* and *TRP-2*. The results are consistent with the increase in tyrosinase activity and melanogenesis induced by alternol.

Discussion

Malignant melanoma accounts for 80% of all deaths from skin cancer (29). At present, an increased proliferation capacity to metastasize and broad spectrum of associated genetic and epigenetic changes contribute to the resistance of melanoma therapy. The inhibitory effects of multiplication and tumorigenicity are a significant marker of induced differentiation. Although surgical resection and chemotherapy play a key role

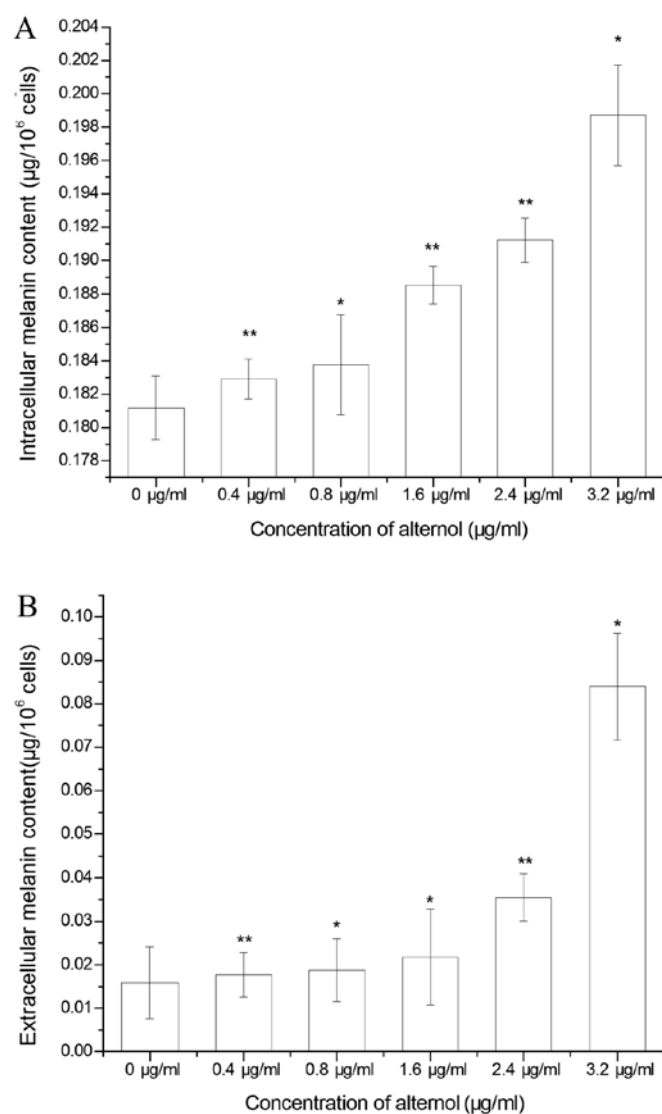


Figure 6. Alternol pretreatment induces melanogenesis of B16F0 cells. B16F0 cells were treated with alternol for 48 h. The melanin content was measured as described previously (23,24). Results were calculated as the amount of intracellular melanin per cell (A) and extracellular melanin per cell (B). Results are the mean \pm SEM from three separate experiments. * $P < 0.05$, ** $P < 0.01$ vs. the vehicle control-treated cells.

in improving the survival rate, drug resistance, relapse and metastasis remain the main obstacles to the success of cancer treatments. Lack of effectiveness of anti-melanoma therapies makes it necessary to search for new drugs that improve or replace standard chemotherapy. Defect in cell differentiation is the main factor that causes malignant proliferation of tumor cells (30). Cell differentiation is usually accompanied by a low proliferation rate. Thus, differentiation therapy opens a new area in the field of tumor treatment. At present, although the induction of differentiation therapy is an effective method with low damage to the body, most differentiation-inducing agents are toxic. For example, antitumor drugs such as phorbol esters (TPA) and dimethyl sulfoxide (DMSO) have good effects on differentiation induction (31-33), yet they have toxic effects which makes it difficult for clinical application. The aim of our study was to clarify the differentiation-inducing activity of alternol. Alternol obviously induced cancer cell differentiation,

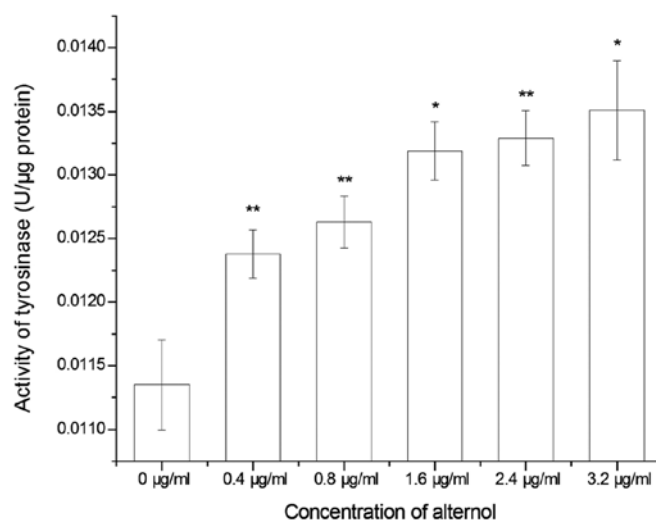


Figure 7. Effects of alternol on tyrosinase activity. Values were normalized based on the protein concentrations. Each experiment was carried out in triplicate. The data are expressed as percentages compared to the controls and are shown as the mean \pm SEM. * $P < 0.05$, ** $P < 0.01$.

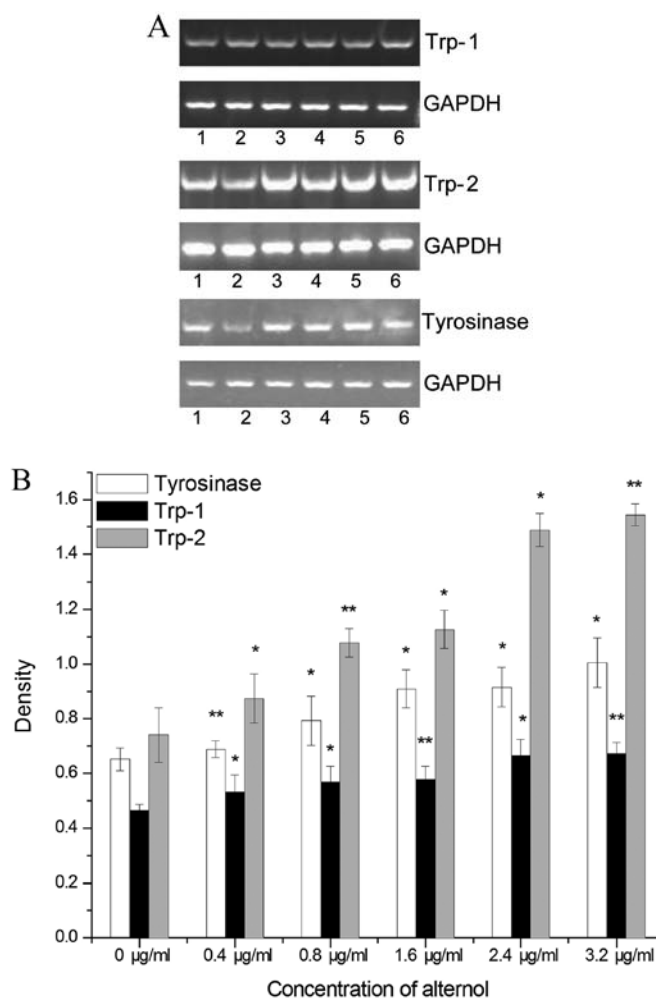


Figure 8. Effects of alternol (lane 1, 0 µg/ml; lane 2, 0.4 µg/ml; lane 3, 0.8 µg/ml; lane 4, 1.6 µg/ml; lane 5, 2.4 µg/ml; lane 6, 3.2 µg/ml) on the expression of tyrosinase, *TRP-1* and *TRP-2*. (A) The expression levels of tyrosinase, *Trp1* and *Trp2* mRNA were analyzed by RT-PCR. (B) Relative expression is shown as normalized to GAPDH in all cells. Bars represent means \pm SEM of three independent experiments * $P < 0.05$, ** $P < 0.01$, vs. the control.

but also had a cytotoxic effect. In future research, we will alter the structure of alternol to obtain analogues with a superior inducing effect and low toxicity. It is imperative to identify novel antitumor drugs which have low toxicity and superior tumor-suppressing effects.

Alternol as a novel compound plays a potential role in the treatment of many types of cancer. In the present study, we treated B16F0 cells with different concentrations of alternol. The results revealed that alternol significantly inhibited the growth of B16F0 cells and the colony formation rate. Marked changes in cell morphology were observed in the B16F0 cells after treatment with alternol. In addition, long outgrowth and dendritic structure became increasingly visible. Both intracellular and extracellular melanin content were evidently increased along with alternol in a dose-dependent manner. Moreover tyrosinase is one of the key enzymes in the melanin biosynthesis of B16F0 cells (34). In addition, tyrosinase-related protein such as TRP-1 and TRP-2 play extremely essential roles in the process of melanin synthesis (35,36). Tyrosinase can catalyze tyrosine into DOPA, and then DOPA is oxidized into dopaquinone; thus the accumulation of melanin pigments is accomplished (37,38). Alternol upregulated the mRNA level of tyrosinase, *TRP-1* and *TRP-2*. All these results indicated that alternol can obviously increase the content of differentiation markers in melanoma cells (39). The mechanism of the proliferation inhibition effect of alternol is mediated by induction of the differentiation of melanoma cells.

Overall, the obtained results suggest that alternol is a potent anti-melanoma agent, reducing proliferation and inducing differentiation of the mouse melanoma B16F0 cell line in a dose-dependent manner. Alternol has good prospects for clinical application. The possibility of synergistic action of alternol with known anticancer drugs warrents further testing. Currently, our findings provide further support for the clinical application of alternol in the inhibition and therapy of cancer.

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