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Alantolactone induces cell apoptosis partially through down-regulation of testes-specific protease 50 expression

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HIGHLIGHTS

- Alantolactone is a novel and efficient inhibitor of TSP50 expression.
- Alantolactone produced cytotoxic effects on the cells which had high levels expression of endogenous TSP50.
- Alantolactone induced cell apoptosis via mitochondrial-dependent pathway.
- Alantolactone triggers cell apoptosis by inhibiting TSP50 expression.

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ABSTRACT

Testes-specific protease 50 (TSP50) is aberrantly expressed in many cancer biopsies and plays a crucial role in tumorigenesis, which make it a potential cancer therapeutic target for drug discovery. Here, we constructed a firefly luciferase reporter driven by the *TSP50* gene promoter to screen natural compounds capable of inhibiting the expression of *TSP50*. Then we identified alantolactone, a sesquiterpene lactone, could efficiently inhibit the promoter activity of TSP50 gene, further results revealed that alantolactone also efficiently inhibited the expression of TSP50 in both mRNA and protein levels. Moreover, we found alantolactone could increase the ratio of Bax/Bcl-2, and activate caspase-9 and caspase-3 in the cancer cells with high expression of TSP50, surprisingly, the same effects can also be observed in the same cells just by knockdown of TSP50 gene expression. Furthermore, our results suggested that overexpression of TSP50 decreased the cell sensitivity to alantolactone-induced apoptosis in those cancer cells. Taken together, these results suggest that alantolactone induces mitochondrial-dependent apoptosis at least partially via down-regulation of TSP50 expression.

1. Introduction

Cancer is a leading cause of death worldwide and the deaths from cancer worldwide are projected to continue to rise to over 13.1 million in 2030 (Ferlay et al., 2010). Until now, conventional cancer therapies including surgery, chemotherapy and radiotherapy, no matter alone or in combination, have poor prognosis and serious side effects (Xu et al., 2006). In contrast to conventional cancer therapies, molecular-targeted therapy has lots of notable advances and becomes a critical focus on cancer therapies. The target molecules that are practical basis for targeted therapy should be essential for the survival and proliferation of cancer cells, amenable to therapeutic targeting, and drugs acting on them should spare normal cells (Ocana et al., 2011).

TSP50 gene was discovered by a hypomethylated DNA fragment isolated from human breast cancer cells in 1999 (Yuan et al., 1999). TSP50 transcripts are not visible in normal tissues except testes, however, it is abnormally reactivated in many breast cancer biopsies and lots of cancer cell lines (Shan et al., 2002; Xu et al., 2004; Zheng et al., 2011). Our previous results suggested that overexpression of TSP50 in TSP50-deficient CHO cells markedly increased cell proliferation and colony formation, and promoted tumor formation in nude mice (Li et al., 2012; Song et al., 2011), moreover, down-regulation of TSP50 induces apoptosis, reduces cell proliferation and colony formation in p19 cells (Zhou et al., 2010), suggesting

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that TSP50, as an oncogene, plays a crucial role in tumorigenesis, and it is necessary for growth of some cancer cells. All this indicated that TSP50 could be a novel drug screening target for cancer therapy.

Traditional Chinese medicine (TCM) is recognized as rich resources for drug discovery in cancer treatment. Of the 121 prescription drugs in use today for cancer treatment, 90 were derived from medicinal plants (Craig, 1999; Wang et al., 2012). Alantolactone, a sesquiterpene lactone, is mainly extracted from the roots of Elecampane (*Inula helenium* L.) which is a traditional Chinese medicinal herb officially listed in some European pharmacopeias (Stojakowska et al., 2006; Trendafilova et al., 2010). It has been reported that alantolactone possesses an inhibitory activity for cell growth in some kinds of cancer cell lines (Konishi et al., 2002; Lawrence et al., 2001) and induce cell apoptosis in human hepatoma and glioblastoma cells by modulating Bcl-2 family proteins and caspases activation or by GSH depletion, ROS generation, and mitochondrial dysfunction (Khan et al., 2012; Lei et al., 2012). Furthermore, results from Khan suggested that alantolactone did not induce significant hepatotoxicity and nephrotoxicity in vivo (Khan et al., 2012).

Here, we established a firefly luciferase reporter screening system driven by TSP50 promoter, and screened over 300 purified compounds derived from traditional Chinese medicine, and we found that alantolactone effectively inhibited TSP50 protein levels. Further results showed that alantolactone could induce mitochondrial-dependent apoptosis in cancer cells that expressed high levels of TSP50, but had no significant effects on cancer cells that expressed low levels of TSP50. These results demonstrated that TSP50 was a new cancer therapeutic target and alantolactone was a potential antitumor drug for tumors expressing high levels of TSP50.

2. Materials and methods

2.1. Cell lines and cell culture

Human embryonic kidney (HEK293T) cells, human hepatocyte (L02) cells, human liver carcinoma (HepG2) cells, and human breast cancer (MDA-MB-231) cells were cultured in DMEM (Dulbecco's modified Eagle's medium; Gibco, Invitrogen, USA). The human hepatocarcinoma (SMMC7721) cells, human liver cancer (H7402) cells, human gastric carcinoma (SGC7901) cells and human lung carcinoma (A549) cells were cultured in RPMI 1640 (GIBCO, Invitrogen, NY). All cells were supplemented with 10% FBS (fetal bovine serum; TBD Science, Hangzhou, China), 100 units/mL penicillin and 100 μg/mL streptomycin (Ameresco, USA) at 37 °C with 5% CO_2.

2.2. Plasmid constructs and transfection

The pRNAT-U6.1/Hygro vector (GenScript) was used for DNA vector-based shRNA synthesis. The TSP50 shRNA expression vectors were constructed as described previously (Song et al., 2011). All plasmids were transfected using Lipofectamine 2000 (Invitrogen, NY), according to the manufacturer's instructions. The expression plasmid pcDNA3-TSP50 was prepared in our laboratory.

2.3. Screening of potential inhibitors of TSP50 expression

In primary screening assay, HEK293T cells were plated at 2×10^6 cells/well in a 6-well plate. After 24 h, cells were transfected with 2 μg of pGL3-TSP50 (pGL3-1237/+454) (Wang et al., 2008) plasmids using the calcium phosphate cell transfection kit (Beyotime, Hainan, China), and were maintained in DMEM. After 24 h, the transfected cells were plated onto the 96-well plates at a density of 5×10^3 cells/well. 24 h later, cells were treated with natural compounds at final concentrations of 5 μg/mL in DMEM containing 3% FBS (v/v) (to reduced the complex interference caused by the composition of serum) for 24 h. Luciferase activity was measured as described previously (Sun et al., 2011; Zhang et al., 2010a).

In secondary screening assay, HEK293T cells were plated at a concentration of 5×10^4 cells/well in a 24-well plate. After 24 h, cells were transfected with 1 μg of pGL3-TSP50 plasmids or 1 μg of pGL3-basic vector plasmids per well plus 0.1 μg of pCMV-β-galactosidase plasmids using the calcium phosphate cell transfection kit according to the instructions of manufacturer. The cells were incubated for 24 h and then cells were treated with the identified compounds at final concentrations of 5 μg/mL or DMSO (the negative control) for 24 h.

2.4. RNA extraction and RT-PCR

MDA-MB-231 cells were plated at a concentration of 5×10^5 cells/well in a 6-well plate. After culture of 24 h, cells were treated with alantolactone (5 μg/mL) in 2 mL DMEM containing 3% FBS (v/v) for another 24 h. Total RNA was extracted from the cells. RNA extraction and RT-PCR were performed as described previously (Wang et al., 2010).

2.5. Protein extraction and western blotting analysis

To determine the expression of associated proteins, cytosolic and nuclear extracts were prepared and Western blotting was performed as described previously (Lin et al., 2012). Mouse monoclonal antibody against Bcl-2 (1:500) and rabbit polyclonal antibody against Bax (1:800) were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Rabbit polyclonal antibodies against cleaved caspase-9 (1:1000) and cleaved caspase-3 (1:1000) were from Cell Signaling (Beverly, MA, USA). Anti-TSP50 monoclonal antibody (1:500) was prepared in our laboratory (Liu et al., 2009). Antibody–antigen complexes were incubated with horseradish peroxidase (HRP)-conjugated secondary antibodies (goat anti-rabbit IgG or goat anti-mouse IgG, 1:1000), followed by detection using ECL-Plus kit (Beyotime, Hainan, China).

2.6. MTT assay

Cells were seeded into 96-well plates for 24 h at 37 °C and then treated with compounds in the presence of 3% FBS (v/v) medium for 48 h at 37 °C. After that, 20 μL MTT solution (5 μg/mL in PBS; Sigma, St. Louis, MO, USA) was added to each well and incubated for 4 h at 37 °C before removal of the culture medium. 150 μL DMSO was then added and shaken for 30 min at room temperature. Cell viability was determined by measuring the absorbance at 570 nm by plate reader (Bio-Rad, CA, USA). The inhibition rate of compounds on each cell lines was evaluated, and IC_{50} and IC_{10} values were calculated by SPSS method. All determinations were carried out in triplicate.

2.7. Cell membrane and nuclear staining

The cells were plated into 96-well plates and allowed to grow for 24 h. After treatment with compounds, the cells were washed three times with phosphate-buffered saline (PBS) and then fixed with Paraformaldehyde (4% in PBS, pH 7.4; Sigma) for 20 min at room temperature. After washing three times with PBS, the fixed cells were stained with DiI (5 μM in PBS; Beyotime, Hainan, China) and DAPI (0.5 μg/mL in PBS; Beyotime, Hainan, China) for 20 min in a dark environment at 37 °C followed by washed three times with PBS, then the cells were analyzed under an Olympus BX50 fluorescence microscope (Olympus, Tokyo, Japan).

2.8. Apoptosis analysis

The cells were plated into 96-well plates and allowed to grow for 24 h. After treatment with compounds, the cells were then stained with TUNEL (In Situ Cell Death Detection Kit, Fluorescein, Roche, Switzerland) following manufacturer's instructions. After washing three times with PBS, cells were stained with DAPI following the protocol described above. The images were acquired using Olympus BX50 fluorescence microscope.

2.9. Statistical analysis

Data were analyzed using the SPSS software. One-way analysis of variance (ANOVA) followed by *post hoc* Tukey's HSD multiple comparisons were carried out. Experiments were repeated at least three times with two replicates per sample. Luciferase activity was normalized by β-galactosidase activity. The significance level was set as $^*P < 0.05$ and $^{**}P < 0.01$. Error bars denote SD unless stated otherwise.

3. Results

3.1. Screening of TSP50 expression inhibitor

It has been shown that TSP50 is aberrantly expressed in many cancer cells (Song et al., 2011) and it plays an important role in tumorigenesis (Xu et al., 2004), which make it a possible drug screening target for cancer therapy. In order to obtain new compounds that selectively target TSP50, we established a firefly luciferase reporter screening system driven by TSP50 promoter and screened potential candidate inhibitors capable of inhibiting the expression of *TSP50* from 300 natural compounds. Alantolactone (see structure in Fig. 1C) was found to have significant inhibitory