·Original Article·

**A novel sesquiterpene Hirsutanol A induces autophagical cell death in human hepatocellular carcinoma cells by increasing reactive oxygen species**

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**Abstract**

**Background and Objective:** Hirsutanol A is a novel sesquiterpene compound purified from fungus *chondrostereum* sp in *Sarcophyton tortuusum*. Its pharmacologic effect has not been reported yet. This study aimed to investigate cytotoxic effect of Hirsutanol A on hepatocellular carcinoma (HCC) cells and its mechanism. **Methods:** Hep3B cells were treated with different concentrations of Hirsutanol A. Cell proliferation was detected by MTT assay. The protein expression of LC3 was determined by Western blot. The generation of reactive oxygen species (ROS) was monitored by flow cytometry. **Results:** Hirsutanol A significantly inhibited proliferation of Hep3B cells with 50% inhibition concentrations (IC50) of 14.54, 6.71, and 3.59 μmol/L when exposed to Hirsutanol A for 24, 48, and 72 h, respectively. Incubation of Hep3B cells with Hirsutanol A markedly increased the level of ROS and the autophagy marker MAP-LC3 conversion from type I to type II. Pre-incubation with an antioxidant N-acetyl cysteine (NAC) decreased the level of ROS, and reduced MAP-LC3 I–II conversion, and suppressed cell death. Blocking autophagy with a specific autophagy inhibitor 3-methyladenine (3-MA), the cytotoxic effect of this compound was attenuated. **Conclusion:** Hirsutanol A has potent cytotoxic effect, and can induce autophagic cell death via increasing ROS production.

**Keywords:** Hirsutanol A, autophagic cell death, LC3, ROS

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There are abundant of biological resources in ocean and marine organisms which contain a lot of novel compounds. With the reduction of the scope of development and exploration of terrestrial biological resources of medicine, people have to cast their sights on the ocean with a hope to find and develop antitumor drugs with low toxicity and high efficacy from marine organisms. Hirsutanol A is a sesquiterpene compound which is isolated from fungus *chondrostereum* sp in *Sarcophyton tortuusum* from the South China Sea coral, the chemical structure of which has been reported only once and its pharmacological activity has not been reported yet. We have detected the cytotoxic activity of six compounds extracted from marine organisms and found that Hirsutanol A had obvious cytotoxicity. Reactive oxygen species (ROS), a class of single electron reduction products of oxygen in vivo, are generated by electrons which leak from respiratory chain and consume about 2% of the oxygen before electrons are transmitted to the terminal oxidase. ROS can induce apoptosis and autophagy in tumor cells. On the one hand, autophagy can provide energy for cells by degrading their own damaged organelles to protect cells from death. On the other hand, excessive autophagy can cause autophagic cell death. Due to a long-term high-level of ROS, the redox system of tumor cells are more vulnerable than that of normal cells. Treating cells with the drugs which can increase ROS levels would selectively kill tumor cells sparing normal cells. The redox system of cells...
is considered to be a new target for antitumor drugs\textsuperscript{2,3}. Hirsutanol A has an unsaturated carbonyl. Analyzing the chemical structure, the compound has a strong oxidative effect. We speculated that the compound may induce apoptosis or autophagic cell death of tumor cells by elevating ROS. This study mainly investigated the effect of Hirsutanol A on ROS levels in hepatocellular carcinoma (HCC) cells and autophagic cell death caused by elevating ROS levels. It provided a basis for further clarification of the anti-tumor mechanisms of Hirsutanol A and provided novel lead compound for developing antitumor drugs with high efficacy and low toxicity targeting the redox system.

**Materials and Methods**

**Cell lines and cell culture**

Human HCC cell lines Hep3B, Bel-7402 nasopharyngeal carcinoma cell line CNE2, lung cancer cell line A549, colon cancer cell lines SW480 and LOVO, and cervical cancer cell line HeLa were cultured with RPMI-1640 culture medium containing 10% fetal bovine serum (FBS, Gibco Company) at 37°C in 5% CO\textsubscript{2}. All experiments were carried out with cells in logarithmic growth phase.

**Materials and reagents**

Methyl thiazolyl tetrazolium (MTT), CM-H2DCF-DA, autophagy inhibitor 3-methyladenine (3-MA), and anti-oxidant N-acetyl cystein (NAC) were purchased from Sigma Company; chemiluminescent agent ECL was purchased from Cell Signal Company; LC3 antibody was purchased from Novus Biological Company; GAPDH, anti-rabbit and anti-mouse secondary antibodies were purchased from Santa Cruz Company; cell lysate solution was purchased from Upstate Biotechnology Company; BCA Protein Assay Kit was purchased from Pierce Inc.; Hirsutanol A was isolated by College of Chemistry and Chemical Engineering, Sun Yat-sen University, whose chemical structure is shown in Figure 1.

**MTT assay**

Hep3B, Bel-7402, CNE2, A549, SW480, LOVO, and HeLa cells in logarithmic phase were seeded into 96-well plates at a density of 6000–10000 cells per well in 195 µL, each with 4 parallel wells, then 5 µL of Hirsutanol A at different concentrations was added to each well; Hep3B cells were pretreated with 10 mmol/L 3-MA\textsuperscript{4,5} or 1 mmol/L NAC\textsuperscript{6} for 1 h followed by incubation at 37°C for 72 h. A total of 10 µL of 5 mg/mL MTT was added 4 h before the termination of the experiment. Afterwards, culture medium were removed and 100 µL of DMSO was added. A value of each well at a wavelength of 570 nm was detected with the microplate reader (BIO-RAD Model 550) after the crystal was dissolved. The rates of cell growth inhibition were calculated. The 50% inhibitory rates (%) were then calculated by the BLISS method: 50% inhibitory rate = (1 – the average A value of treatment group / the average A value of the control group) × 100%.

**Western blot**

Hep3B cells were diluted to 3.0 × 10\textsuperscript{5} per mL and seeded into 6-well plates. Hep3B cells were treated with 20 µmol/L Hirsutanol A for different times, or pretreated with NAC for 1 h and then treated with 20 µmol/L Hirsutanol A for 24 h. Cells were colleted and washed with PBS twice, 100 µL cell lysate was added and centrifuged at 14 000 r/min for 10 min. Proteins were quantified. After loading buffer was added to cell lysate, samples were denatured for 10 min at 95°C. SDS polyacrylamide gel (10% - 15%) electrophoresis was carried out and the protein was transferred to PVDF membrane. After blocked with 5% non-fat milk, the primary and secondary antibodies were added successively. The membrane was incubated at room temperature for 2 h and washed with TBST buffer 3 times for 10 min each. ECL was added, then the membrane was put in the cassette and pressed with a film 2–5 min followed by developing and fixing.

**Flow cytometry**

Hep3B cells were diluted to 2.0 × 10\textsuperscript{5}/mL and seeded into 6-well plates with 2 mL in each well. Cells were treated with 20 µmol/L Hirsutanol A for different times, or treated with Hirsutanol A at different concentrations for 24 h. Then, 1 µmol/L (final concentration) CM-H2DCF-DA fluorescent dye was added and the cells were incubated at 37°C in dark for 1 h. After washed in 4°C PBS twice, cells were centrifuged and resuspended with PBS. Flow cytometer (BECKMAN- COULTET FC500) with FACS Calibur system with CellQuestPro was used to detect intracellular ROS.
levels[7].

Statistical analysis

Data are presented as mean ± standard deviation (SD) and were analyzed by student’s t test with SPSS 11.0 analysis software. A P value of < 0.05 was considered as significantly different.

Results

Inhibition of tumor cell proliferation by Hirsutanol A

Hirsutanol A was found to have significant cytotoxicity and the IC_{50} values were 12.43 μmol/L for CNE2 cells, 11.81 μmol/L for A549 cells, 24.23 μmol/L for Bel-7402 cells, 12.04 μmol/L for SW480 cells, 19.89 μmol/L for LOVO cells, and 32.81 μmol/L for HeLa cells. The IC_{50} values of Hirsutanol A for Hep3B cells were 14.54 μmol/L at 24 h, 6.71 μmol/L at 48 h, and 3.59 μmol/L at 72 h (Figure 2).

Hirsutanol A induced of LC3 from type I to type II

When Hep3B cells were treated with 20 μmol/L Hirsutanol A for 1 h, 6 h, 12 h, and 24 h, respectively, Western blot results showed that the conversion of LC3 from type I to type II was increased in a time-dependent manner (Figure 3).

Hirsutanol A induced an increase of ROS levels in Hep3B cells

When Hep3B cells were treated with 5, 10, and 20 μmol/L Hirsutanol A for 24 h, ROS levels increased significantly in a concentration-dependent manner. When Hep3B cells were treated with 20 μmol/L Hirsutanol A for 1 h, ROS levels increased significantly; when treated for 12 and 24 h, ROS levels decreased to some extent, but still significantly higher than those of the control group (P < 0.05) (Figure 4).

NAC, a potent antioxidant prevented Hirsutanol A-induced autophagic cell death in Hep3B cells

When Hep3B cells were pretreated with 1 mmol/L NAC for 1 h and treated with Hirsutanol A for 3 h, flow cytometry results showed that NAC completely prevented the increase of ROS induced by Hirsutanol A (Figure 5). When Hep3B cells were pretreated with 1 mmol/L NAC for 1 h and treated with Hirsutanol A for 72 h, MTT assay results showed that NAC significantly reduced Hirsutanol A-induced cell death (Figure 6). Western blot results showed that the conversion of LC3 from type I to type II in Hep3B cells was inhibited significantly after pretreatment of NAC (Figure 7).
Figure 4  Effect of Hirsutanol A on cellular ROS level
Hep3B cells were treated with various concentrations of Hirsutanol A for 24 h (A) or treated with 20 μmol/L Hirsutanol A for indicated times (B), then stained with CM-H2DCF-DA for 30 min. The cellular ROS level was monitored by flow cytometry.

Figure 5  NAC prevented the increase of cellular ROS level induced by Hirsutanol A
Hep3B cells were preincubated with NAC for 1 h, then exposed on Hirsutanol A for 3 h. The cellular ROS level was monitored by flow cytometry.

Figure 6  NAC inhibited cell death induced by Hirsutanol A
Hep3B cells were preincubated with NAC for 1 h followed by treatment with Hirsutanol A for 72 h, then detected by MTT assay.

**Blockage of autophagy by 3-MA decreased Hirsutanol A-induced cell death**

Hep3B cells were pretreated with 10 mmol/L 3-MA for 1 h and then treated with different concentrations of Hirsutanol A for 72 h. MTT assay showed that 3-MA significantly reduced Hirsutanol A-induced cell death (P < 0.05) (Figure 8).
Chemotherapy is one of the three major treatment means of tumor. Therefore, developing new and effective anticancer drugs is still the main strategy to conquer tumor. Due to the particularity of their living environments (high pressure, hypoxia, high salt, and so on), marine organisms often contain a large number of metabolites with novel structure and significant physiologic activity. Therefore, to find natural medicine with high-efficacy and low toxicity from marine organisms has attracted increasing international interest. A large number of natural products with significant antitumor, antibacterial, antiviral, and antioxidant activities have been isolated, most of which have never been found in terrestrial organisms. For example, the leads of antitumor and antiviral drugs cytosine arabinoside A and C, which were widely used clinically, were isolated from the sponge *Cryptotethya* sp.[8]. We have screened 6 compounds isolated from marine organisms in terms of anti-tumor effect and found that Hirsutanol A has obvious cytotoxicity. Hirsutanol A is a sesquiterpene compound isolated from fungus *chondrostereum* sp in *Sarcophyton tortuosum* in the South China Sea coral. It is isolated from a new microbial strain, the pharmacological activity has not been reported. Therefore, we further explored its antitumor effects and its mechanisms.

ROS, a class of single electron reduction products of oxygen in vivo, are generated by electrons which leak from respiratory chain and consume about 2% of the oxygen before electrons are transmitted to the terminal oxidase, including one-electron reduzate—oxygen superoxide anions (O2-), two-electron reduzate—hydrogen peroxide (H2O2), three-electron reduzate—hydroxyl radical (OH), nitric oxide, and so on. The generation of ROS is closely associated with tumorigenesis and treatment. ROS is a two-edged sword. On the one hand, an abnormal increase of ROS can promote tumorigenesis; on the other hand, excessive increase of ROS can induce apoptosis and autophagic death of tumor cells, result in cancer cell growth inhibition[9,10]. In the present study, we found that Hirsutanol A could induce Hep3B cells to produce ROS and ROS scavenger NAC could significantly prevent Hirsutanol A-induced cell death.

LC3 is the mammalian homologue of yeast Atg8, locates on the membrane surface of preautophagosomes and autophagic vacuoles. It is a common marker for autophagic vacuolar membranes. Newly synthesized LC3 in cells becomes cytoplasmic soluble LC3-I after process. After ubiquitin-like modification, LC3-I combines with phosphatidyl ethanolamine (PE) which is located on the membrane surface of autophagic vacuoles, known as LC3-II. The content of LC3-II is positively related to the number of autophagic vacuoles. Therefore, changes of the content of LC3-II reflect the autophagic activity of the cells to some extent[11,12]. Hirsutanol A can induce LC3-II expression. After treatment with ROS scavenger NAC, the conversion of LC3 from type I to type II reduced, suggesting that Hirsutanol A induced HCC cells to undergo autophagy by increasing ROS. PI3K is the enzyme family which
catalyzes phosphorylation of inositol group of membrane phosphoinositide. According to structural subunits and characteristics of substrates, PI3K can be divided into types I, II and III, of which type III phosphoinositide 3 kinase (PI3KC3) is a mammalian homologue of yeast Vps34. PI3KC3 forms compounds with Beclin 1, Vps15, and so on, and plays a necessary role in the initial stage of autophagic vesicle formation and the transport process of transferring cell contents to the lysosome\textsuperscript{[13]}. PI3KC3 inhibitor 3-MA can inhibit the occurrence of autophagy effectively in the initial stage of autophagy. When blocked autophagy with 3-MA, Hirsutanol A-induced cell death significantly reduced, suggesting that Hirsutanol A induced autophagic cell death of Hep3B cells by the induction of ROS. Hirsutanol A has a new chemical structure with antitumor activity. Its antitumor mechanisms in vitro and in vivo remain to be studied in the future.

References


