Ergosta-4,6,8(14),22-tetraen-3-one induces G2/M cell cycle arrest and apoptosis in human hepatocellular carcinoma HepG2 cells

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1. Introduction

Hepatocellular carcinoma (HCC) is a worldwide major health problem, with an estimated incidence ranging from 500,000 to 1 million new cases annually\textsuperscript{[1]}. Currently, the clinical treatments include surgery, radiotherapy, and other therapies. However, the great majority of cases, especially for advanced-stage cancer, is still treated with conventional chemotherapy. The efficacy of drugs is often hampered by a range of adverse side-effects imposed on patients. Hence, there is a great demand for the development of new therapeutic drugs against this disease.

Background: Mushrooms have been used in Asia as traditional foods and medicines for a long time. Ergosta-4,6,8(14),22-tetraen-3-one (ergone) is one of the well-known bioactive steroids, which exists widely in various medicinal fungi such as Polyporus umbellatus, Russula cyanoxantha, and Cordyceps sinensis. Ergone has been demonstrated to possess cytotoxic activity. However, the molecular mechanisms by which ergone exerts its cytotoxic activity are currently unknown.

Methods: In the present study, ergone possessed a remarkable anti-proliferative activity toward human hepatocellular carcinoma HepG2 cells. We assayed the cell cycle by flow cytometry using PI staining; investigated the exposure of phosphatidylserine at the outer layer of the cytoplasmic membrane by the FITC-annexin V/PI staining; observed the nuclear fragmentation by Hoechst 33258 staining and studied the protein expression of Bax, Bcl-2, p-53, procaspase-3, -8, -9, PARP and cleaved PARP by Western blotting analysis.

Results: Cells treated with ergone showed typical markers of apoptosis: G2/M cell cycle arrest, chromatin condensation, nuclear fragmentation, and phosphatidylserine exposure. Furthermore, PARP-cleavage; activation of caspase-3, -8, -9; up-regulation of Bax and down-regulation of Bcl-2 were observed in HepG2 cells treated with ergone, which show that both the intrinsic and extrinsic apoptotic pathways are involved in ergone-induced apoptosis in HepG2 cells. Ergosta-4,6,8(14),22-tetraen-3-one induces G2/M cell cycle arrest and apoptosis in HepG2 cells in a caspase-dependent manner.

General Significance: In this study, we reported for the first time that ergone-induced apoptosis through activating the caspase. These results would be useful for the further utilization of many medicinal fungi in cancer treatment.

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Mushrooms have been used in Asia as traditional foods and medicines for a long time. There are various classes of primary and secondary metabolites in mushrooms and they exhibit significant antimicrobial, antiviral, and antitumor activities\textsuperscript{[2]}. Therefore, they represent a valuable source of novel chemotherapeutic agents. Despite of the potential in drug development, only a few bioactive metabolites from mushrooms have been reported so far as compared with those from the higher plants and microbes. Furthermore, the underlying mechanisms for the antitumor function of bioactive metabolites from mushrooms are also far from being understood.

During the search of anti-cancer agents from high fungi, the n-hexane extract of the dried sclerotia of Polyporus umbellatus (Pers.) Fries (Polyporaceae) was found to show significant anti-proliferative effect on human hepatoma HepG2 cells. Chromatographic fractionation of the extract guided by biological assays prompted us to purify the active constituent which was identified as ergosta-4,6,8(14),22-tetraen-3-one (ergone) in our previous study\textsuperscript{[3]}. Ergone is one of the well-known
bioactive steroids, which exists widely in various medicinal fungi such as *P. umbellatus* [4–6], *Russula cyanoxantha* [7], *Cordyceps sinensis* [8]. Ergone has been reported to possess cytotoxic activity [9], diuretic activity [10], inhibitory activity of nitric oxide production [11] and immunosuppressive activity [12].

In this study, we assessed the molecular mechanisms and inhibitory effects of ergone using human hepatocellular carcinoma HepG2 cells. Our results showed that ergone inhibited the growth of HepG2 cells in both dose- and time-dependent manners. Furthermore, we found that ergone showed significant antitumor effects by inducing G2/M phase arrest and apoptosis. We also found that ergone-induced apoptosis by activating caspases via both intrinsic and extrinsic pathways. In addition, the ratio of Bax/Bcl-2 markedly increased in cells treated with ergone. These results are significant as they provide an insight into the molecular mechanism of ergone which might be a potent chemotherapeutic agent for the treatment of liver cancer.

### 2. Materials and methods

#### 2.1. Chemicals

The standard of ergone was isolated by the authors from *P. umbellatus* and also synthesized by chemical method (Fig. 1). The procedures for isolation, purification and synthesis of ergone were described in our previous studies [3,10]. Its structure was characterized by chemical and spectroscopic methods (¹H NMR, ¹³C NMR and MS) and compared with those found in the literature [13,14]. Its purity was evaluated to be above 99% (HPLC and spectral analysis) and the structure of the compound is shown in Fig. 1.

RPMI 1640, 3-[4,5-Dimethylthiazol-2-yl]2,5-diphenyltetrazolium bromide (MTT), propidium iodide (PI), Hoechst 33258, dimethylsulfoxide (DMSO), FITC-labeled annexin V, fetal bovine serum, RNase A, Triton X-100, Aprotinin, Leupeptin, phenylmethylsulfonyl fluoride, alkaline phosphatase, 5-bromo-4-chloro-3-indolyl-phosphate (BCIP), nitroblue tetrazolium (NBT) and Antibodies (Bax, Bcl-2, p-53, procaspase-3, -8, -9, PARP, cleaved PARP and ß-actin) were purchased from Sigma Chemical Co., Ltd (St. Louis, MO, USA). Penicillin, streptomycin, ethanol, tris buffer (pH 7.4) containing 1% RNase A and stained with propidium iodide (PI) (5 μg/ml). Distribution of cells with different DNA contents was determined by a flow cytometer (Epics Elite Flow Cytometer, Beckman Coulter) and the data were analyzed by multi-cycle DNA content and cell cycle analysis software (Modfit 3.0).

#### 2.2. Cell culture and cytotoxicity assay

HepG2 was obtained from The Fourth Military Medical University, Xi’an, China. The cells were cultured in RPMI 1640 medium containing 10% heat-inactivated fetal bovine serum, 100 U/ml penicillin and 100 μg/ml streptomycin. Cultures were maintained in a humidified incubator at 37 °C in an atmosphere of 5% CO₂ and 95% air until they reached confluence. The cytotoxicity of ergone was measured by MTT assay [15]. Briefly, HepG2 cells were placed in 96-well culture plates (1 × 10⁴ cells/well), respectively, and allowed to attach for 12 h before treatment. The cells were then treated with various concentrations of ergone (0, 5, 10, 15 and 20 μg/ml) continued for three different time durations (6, 12 and 24 h). The optical density (OD) in control and drug-treated wells was measured in a microplate reader (BIO-TEK) at a test wavelength of 570 nm. The cytotoxicity of ergone was expressed as IC50 (concentration required for 50% growth inhibition).

#### 2.3. Flow cytometric analysis of cellular DNA content

Cells (1 × 10⁶/well) were treated with different concentrations of ergone (0, 5, 10 and 20 μg/ml) for 12 h at 37 °C and collected and fixed in 70% ethanol at 4 °C overnight. Subsequently, cells were treated with Tris buffer (pH 7.4) containing 1% RNase A and stained with propidium iodide (PI) (5 μg/ml). Distribution of cells with different DNA contents was determined by a flow cytometer (Epics Elite Flow Cytometer, Beckman Coulter) and the data were analyzed by multi-cycle DNA content and cell cycle analysis software (Modfit 3.0).

#### 2.4. Annexin V-FITC/PI staining experiment

Flow cytometry was used to determine the externalization of phosphatidyserine by FITC-labeled annexin V and PI, as previously described [16] with minor modification. Briefly, cells (1 × 10⁶/well) after 12 h treatment with different concentrations of ergone (0, 5, 10 and 20 μg/ml) were washed with PBS and then stained with 500 μl labeling solution containing FITC-labeled annexin V and PI for 15 min at room temperature. Subsequently, the samples were subjected to Epics Elite flow cytometer and the data were analyzed using software (Modfit 3.0). The cells in early stages of apoptosis were annexin V positive and PI negative, whereas those in late stages of apoptosis were both annexin V and PI positive.

#### 2.5. Cell morphology observation (Hoechst 33258)

Nuclear fragmentation was visualized by Hoechst 33258 staining of apoptotic nuclei. The cells were exposed to ergone with the concentrations of 0, 5, 10 and 20 μg/ml, respectively. After 12 h exposure, cells were harvested and washed with PBS twice, then fixed with a mixture of acetic acid–ethanol (1:3) for 10 min before deposition on polylysine-coated coverslips. Then the samples were permeabilized with 0.1% Triton X-100 for 5 min at room temperature and stained with 1 μg/ml Hoechst 33258 for 30 min at 37 °C. Finally, the slides were mounted with glycerol–PBS and viewed with an Olympus FV1000 fluorescence microscope (Tokyo, Japan).

#### 2.6. Western blotting analysis

Cells (1 × 10⁶/dish) were seeded in 10 cm dishes. After 12 h incubation, cells were treated with 0, 5, 10 and 20 μg/ml of ergone for 12 h. For total cell protein extracts, control and treated cells were washed in PBS; suspended in lysis buffer containing 50 mM Tris–HCl (pH 7.5), 1% NP-40, 2 mM EDTA, 10 mM NaCl, 10 μg/ml Aprotinin, 10 μg/ml Leupeptin, 1 mM DTT, 0.1% SDS, 1 mM phenylmethylsulfonyl fluoride and placed in ice for 30 min. After centrifugation for 15 min at 4 °C, the supernatant was collected. The extraction and isolation of nuclear fraction were performed according to the method of Levites et al. [17]. Protein concentrations were determined using the BCA assay (Pierce, Rockford, IL, USA).

The Western blotting assay was performed as described previously [18]. For Western blot analysis, equal amounts of proteins (30 μg) were separated by SDS–polyacrylamide gel electrophoresis and transferred to a nitrocellulose membrane. The blot was blocked in blocking buffer (5% non-fat dry milk/1% Tween 20 in PBS) for 1 h at room temperature and incubated with appropriate primary antibodies in blocking buffer overnight at 4 °C. The blot was then incubated with appropriate secondary antibodies and visualized by enhanced chemiluminescence (ECL). The Western blotting assay was performed as described previously [19]. For Western blot analysis, equal amounts of proteins (30 μg) were separated by SDS–polyacrylamide gel electrophoresis and transferred to a nitrocellulose membrane. The blot was blocked in blocking buffer (5% non-fat dry milk/1% Tween 20 in PBS) for 1 h at room temperature and incubated with appropriate primary antibodies in blocking buffer overnight at 4 °C. The blot was then incubated with appropriate secondary antibodies and visualized by enhanced chemiluminescence (ECL).
secondary antibody alkaline phosphatase (AP) conjugated and detected in 10 ml of AP buffer containing 33 μl of BCIP and 66 of NBT at room temperature for 10–20 min and then photographed. ß-actin was used as a loading control.

2.8. Statistical analysis

In all experiments, data were expressed as means± standard deviation (SD). A significant difference from the respective controls for each experimental test condition was assessed using Student’s unpaired t-test, with P values <0.01 or <0.05 being regarded as statistically significant.

3. Results

3.1. Cytotoxicity of ergone on HepG2 cells

The cytotoxicity of ergone on HepG2 cells was assessed by the MTT assay. Ergone treatment exhibited a remarkable inhibition on the survival of HepG2 cells dose- and time-dependently. From Table 1, the IC50 values were calculated as 15.6, 11.8 and 10.0 μg/ml in cells with 6, 12 and 24 h treatment, respectively. In addition, our previous experiment demonstrated that ergone showed more selective cytotoxic activity to the HepG2 cells than normal human umbilical vein endothelial cell line (HUVEC) [3].

3.2. Ergone induces G2/M phase cell cycle arrest in HepG2 cells

To determine whether apoptosis is involved in the anti-proliferation effect of ergone, HepG2 cells were treated with different concentrations of ergone for 12 h and subjected to flow cytometry analysis for apoptotic activity. Results showed that a typical sub-G0 peak, representing the apoptotic cell population, was detected at different concentrations of ergone. The percentage of apoptotic HepG2 cells of the cultures treated with 0, 5, 10 and 20 μg/ml of ergone was 0, 2.9, 12.8 and 14.2%, respectively (Fig. 2A). Cell cycle distributions are shown as histograms in Fig. 2B. These results indicate that ergone can induce a G2/M phase cell cycle arrest in HepG2 cells, which is associated with the inhibitory effects of ergone against HepG2 cells.

3.3. Effects of ergone on apoptosis of HepG2 cells

In attempt to elucidate whether the loss in cell viability induced by ergone was associated with apoptosis, ergone-induced apoptosis was further performed by annexin V/PI staining. During the early stages of apoptosis, cells display PS on their outer cell membranes, which is readily detectable by annexin V. During the later stages of apoptosis, as the plasma membrane becomes increasingly permeable, PI can move across the cell membrane to bind to cellular DNA. PI provides a means to identify cells that have lost membrane integrity caused by necrosis. In the present study, our results indicated that the proportion of annexin V/PI-stained cells signifying both the early and late apoptotic cells increased with the different concentration of ergone applied (Fig. 3). We detected an increase of early apoptotic (annexin V+/PI−) cells dose-dependently in the presence of ergone. The reliability of PS externalization as an apoptosis marker is very limited because apoptotic cells eventually lose their plasma membrane integrity, leading to secondary necrosis, which is characterized by annexin V+/PI+ staining. Since primary necrosis also results in annexin V+/PI+ staining, the two processes are indistinguishable by this method. For this reason, we chose a relatively short time span (12 h) of ergone treatment. Consistent with the above results, Hoechst 33258 staining showed that some of the ergone-treated cells exhibited highly condensed and fragmented nuclei morphology at concentrations of 5, 10 and 20 μg/ml for 12 h, which are typical characteristics of apoptosis (Fig. 4). In contrast, the cells in the culture without ergone showed normal cell nuclei morphology (Fig. 4). These results suggest that induction of apoptosis by ergone involves the anti-cancer activity.

3.4. Induction of apoptosis through both the extrinsic and intrinsic pathways by ergone

Further understanding of the apoptotic phenomenon observed requires evaluation of the protein level of various key initiators and regulators in the apoptotic pathways, both the extrinsic and intrinsic ones. We further examined the involvement of caspases in ergone-mediated apoptosis. Caspase-3 is one of the chief effector caspases, which plays an indispensable role in the execution of morphological and biochemical changes in apoptosis [19]. Therefore, the protein level of procaspase-3 in cells before and after ergone application was investigated by Western blot analysis. As shown in Fig. 5, cleavage of the precursor of caspase-3 was noted with the presence of ergone in a dose-dependent manner. Concurrently, a reduction in the protein level of the pro-forms of upstream initiator caspases, caspase-8 and caspase-9, was also detected. Compared to the controls, caspase-9 and caspase-8 were all activated after treatment for 12 h. Caspase-mediated PARP-cleavage also showed that ergone-induced apoptosis was a dose-dependent process (Fig. 5).

The mitochondrial pathway involves members of the Bcl-2 family that regulate cytochrome c release from the mitochondria. The influence of ergone-mediated HepG2 on the expression of Bax and Bcl-2 was examined. The apoptotic protein Bax was markedly increased while Bcl-2 was markedly decreased by treatment with ergone at concentrations of 5–20 μg/ml (Fig. 5). Thus, the ratio of Bax/Bcl-2, which is crucial for the activation of the mitochondrial apoptotic pathway, increases in cells treated with ergone. These results demonstrate that both the intrinsic and extrinsic apoptotic pathways are involved in ergone-induced apoptosis in HepG2 cells.

Having a key role in cellular responses to various endogenous and exogenous stimulations, p53 serves as a major barrier of carcinogenesis by inducing cell cycle arrest and apoptosis [20]. P53-mediated cell cycle arrest is partly achieved by transcriptional activation of a CDK inhibitor, p21 WAF1/Cip1 [21]. We detected the expression of p53 in HepG2 cells treated for 12 h with ergone. Ergone affect the expression of p53 compared to a vehicle control (Fig. 5). Collectively, these results suggest that ergone induces cell cycle arrest and apoptosis in a p53-dependent manner in HepG2 cells.

4. Discussion

Ergosta-4,6,8(14),22-tetraen-3-one (ergone), which exists widely in medicinal fungi, was found to significantly inhibit the proliferation of human cancer cells in the previous studies. The anti-proliferation effect has also been demonstrated for ergone in human colon carcinoma HT-29 cells, human cervix carcinoma HeLa 229 cells, human liver carcinoma Hep3B cells and human stomach carcinoma AGS cells [9]. Recently, our studies have also demonstrated that ergone exhibited the potent anti-proliferative effect on human
hepatocellular liver carcinoma HepG2 cells, human laryngeal carcinoma Hep-2 cells and human cervical carcinoma Hela cells by cytotoxic activity screening of *P. umbellatus*. Ergone exhibits the most potent anti-proliferative effect on human hepatocellular liver carcinoma HepG2 cells. Meanwhile, the cytotoxic effects of ergone on normal human cells (human umbilical vein endothelial cells) were much smaller than on cancer cells [3]. These results have demonstrated that ergone could be a promising anti-cancer agent based on its cytotoxicity. The anti-cancer activities of natural compounds, including compounds of mushroom origin [22–24], are examined extensively nowadays. Some of these compounds have been shown to induce apoptosis in cancer cells, e.g., grifolin from the mushroom *Albatrellus confluens* and clitocine from the mushroom *Leucopaxillus giganteus* [25,26].

So far, however, no detailed action mechanism for the cytotoxicity of ergone has been described. In the present study, we have characterized the mechanisms by which ergone exerts its inhibitory effects on HepG2 cells by inducing G2/M cell cycle arrest and apoptosis. Our results show that ergone significantly inhibits the proliferation of HepG2 cells in both time- and dose-dependent manners. The IC50 values were 15.6, 11.8 and 10.0 μg/ml for 6, 12 and 24 h ergone treatment, respectively.

Fig. 2. Dose-dependent effect of ergone on cell cycle distribution. (A) HepG2 cells were treated with different concentrations of ergone (0, 5, 10 and 20 μg/ml) for 12 h and analyzed by DNA flow cytometry. (a) 0 μg/ml, (b) 5 μg/ml, (c) 10 μg/ml, (d) 20 μg/ml. (B) Representative histograms for cell cycle distribution in HepG2 cells. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)
In the present study, flow cytometric analysis showed that ergone can cause G2/M phase arrest in a dose-dependent manner and G2/M phase accumulation peaked at 12 h treatment (Fig. 2), suggesting the sequential events of cell cycle arrest followed by apoptosis. We also detected an increase of early or late apoptotic (annexin V/PI-stained) cells dose-dependently in the presence of ergone. Our results

Fig. 3. Annexin V-FITC/PI staining of HepG2 cells treated with different concentrations of ergone for 12 h. (A) HepG2 cells were exposed to different concentrations of ergone (0, 10, 20 and 30 μg/ml) for 12 h at 37 °C in an atmosphere of 5% CO2. Cells collected were subjected to annexin V-FITC/PI staining and analyzed by flow cytometry. (B) Representative histograms show the fractions of cells in early and late stage of apoptosis.
indicated that the proportion of annexin V/PI-stained cells signifying both the early and late apoptotic cells increased with the different concentration of ergone applied (Fig. 3). These data indicated that the cytotoxic effects of ergone may be mediated by the induction of apoptosis on HepG2 cells. The morphological characteristics of apoptosis include membrane blebbing, cellular shrinkage and chromatin condensation and formation of apoptotic bodies [27]. Staining with Hoechst 33258 clearly showed condensed and fragmented nuclei and apoptotic bodies in the treated cells (Fig. 4).

Apoptosis is a genetically regulated biological process with two major pathways: the death-receptor-induced extrinsic pathway and the mitochondria-apoptosome-mediated apoptotic intrinsic pathway [28]. Bcl-2 family proteins have a central role in controlling the mitochondrial pathway. In human, more than 20 members of this family have been identified including proteins that suppress apoptosis (e.g. Bcl-2, Bcl-x1, Bcl-1) and proteins that promote apoptosis (e.g. Bax, Bak, Bad) [29]. The pro-apoptotic proteins and anti-apoptotic proteins of the Bcl-2 family may turn on and off apoptosis because of the formation of heterodimers among these proteins [30–32]. The heterodimerization results in mutual neutralization of the bound pro- and anti-apoptotic proteins. Therefore, the balance between the expression levels of the protein units (e.g. Bcl-2 and Bax) is critical for cell survival or death. The result showed ergone leading to up-regulation of Bax level and concomitantly down-regulation of Bcl-2 level (Fig. 5). Thus, the ratio of Bax/Bcl-2, which is crucial for the activation of the mitochondrial apoptotic pathway, increases in cells treated with ergone.

The caspase family plays an important role in the regulation of apoptosis. Caspase activation is a hallmark of apoptosis induction in response to death-inducing signals originating from cell surface receptors, mitochondria or the endoplasmic reticulum [33]. In particular, activation of caspase-3 plays a central role in the initiation of apoptosis [34]. This enzyme has substrate specificity for the amino acid sequence Asp-Glu-Val-Asp (DEVD) and cleaves PARP. Activation of caspase-3 requires the activation of initiator caspases, such as caspase-8 or caspase-9, in response to pro-apoptotic signals [35]. Caspase-8 is believed to be activated by engaging certain members of the tumor necrosis factor family with death receptors on cell surface; caspase-9 activation involves the mitochondria. In our study, the event is very clear. After 12 h of ergone treatment, PARP-cleavage was observed, and caspase-3, caspase-8 and caspase-9 were all activated in the cells. Our results indicate that both extrinsic and intrinsic pathways are involved in ergone-induced apoptosis. In addition, p53, the tumor suppressor gene, plays a crucial role in causing cell cycle arrest, apoptosis and repair. In the present study, the p53 protein level was also decreased in HepG2 cells treated with ergone, in comparison to the controls. This result suggests that ergone-induced G2/M arrest and apoptosis is a p53-dependent process.

In conclusion, this study showed that ergone was a potent growth inhibitor of HepG2 cells. The growth inhibition was related to induce G2/M arrest and apoptosis in a dose- and time-dependent manner. Ergone-induced cell cycle arrest was associated with reduction in the
protein in a p53-dependent manner. Ergone also caused a marked increase in apoptosis, which was determined by characteristic morphological changes and increased sub-G0 population. Furthermore, increase of Bax/Bcl-2 ratios was observed after HepG2 cells were treated with ergone, which indicated that the mitochondrial pathway was involved in the apoptosis signal pathway. The work has determined ergone as an effective inhibitor of mitogenesis and inducer of apoptosis, which would not only be useful for the further utilization of many medicinal fungi in cancer treatment but also contribute to the development of ergone in treating liver cancer treatment. The next work is to investigate the effect of ergone in vivo for a further development.

Conflict of interest statement

The authors declare that there are no conflicts of interest.

Acknowledgments

This study was supported in part by grants from the National Scientific Foundation of China (Nos. 81001622, 30830087) and NWU Doctorate Dissertation of Excellence Funds of China (No. 09YBY03).

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