Original Article
3-cinnamoyl-4-hydroxy-6-methyl-2H-pyran-2-one (CHP) inhibits human ovarian cell proliferation by inducing apoptosis

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Abstract: Coumarins induce apoptosis by activating mitochondrial pathway and caspase-3-dependent apoptotic pathway. In the present study, we first time investigated the effect of 3-cinnamoyl-4-hydroxy-6-methyl-2H-pyran-2-one (CHP) on induction of apoptosis in human ovarian carcinoma cells. The data from MTT assay revealed a significant inhibitory effect on cell viability at 30 (87%) and 50 µM (74%) concentration of CHP in OVCAR-3 and OVCAR-420 cells, respectively after 72 h. Apoptosis analysis using annexin V/PI double staining followed by flow cytometry showed 59 and 52% binding to annexin V-FITC in OVCAR-3 and OVCAR-420 cells respectively. Propidium iodide (PI) staining and flow cytometry examination indicated a significant increase in percentage of cells in G2/M phase after treatment with CHP compared to DMSO control group. Reactive oxygen species (ROS) assay kit showed increase in levels of ROS. We used rhodamine-123 (Rh-123) staining and flow cytometry assay to determine changes in mitochondrial membrane potential (ΛΨm). The results revealed that CHP significantly decreased MMP to 85.65 ± 1.2443% & 49.78 ± 1.6554% at 10 and 30 μM respectively in OVCAR-3 compared to 95.97 ± 2.1243% in control group. Western blot analysis clearly indicated a significant increase in the expression of Caspase-3, Bax, and release of Cytochrome c and decrease in Bcl-2, CDK1 and Cyclin B1 expression on treatment with CHP. Therefore, CHP may become a potential candidate for the treatment of human ovarian cancers.

Keywords: Coumarins, apoptosis, annexin V-FITC, flow cytometry, mitochondrial membrane potential

Introduction

Among gynecologic cancers, ovarian cancer is the leading cause of death in the United States [1]. The lifetime risk of ovarian cancer for women with family history in the US is estimated 9.4% [2]. Because of absence of symptoms during early stage, ovarian cancer is usually detected at metastatic stage [3]. Only 25% of the epithelial ovarian cancers are detected as stage I of the disease [4]. The currently available treatment regimens and cytoreductive surgery has increased the cure rate of cancer patients at Stage I to 90% [5]. No doubt use of surgery followed by chemotherapy has increased the response rates [6-8] but complications associated with disease progression cause 75% deaths afterwards. Although, cisplatin is the most important antineoplastic agent against a wide variety of solid tumors [9] but the development of drug resistance hinders its efficiency [10]. Thus the demand for novel therapeutic agents is unmet.

The compounds harbouring lactone, 2-pyrone have promising anticancer activities. The main examples of 2-pyrone-containing compounds include coumarins which exhibit anticancer activity [11, 12]. Coumarins induce apoptosis by activating mitochondrial pathway and caspase-3-dependent apoptotic pathway [13, 14]. For instance, 7,8-Dihydroxycoumarin and its analogs have been reported to enhance tumor apoptosis [15-18] by inhibiting expression of anti-apoptotic Bcl-2 and enhancing expression of Bax [19]. Another derivative, psoralidin enhances tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) and necrosis of HeLa cervical cancer cells [20]. Xanthohydroxyletin induces S phase arrest and apoptosis in SGC-7901 gastric cancer cells [21]. RKS262 was reported to inhibit the ovarian cancer cell cycle and promote apoptosis [22]. Coumarin derivatives were also recognised to promote malignancy of pro-apoptotic factors caspase-3 and -9 [23]. Taking cue from the above literature and promising results of preliminary screening,
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we devised an experiment to investigate the role of CHP (Figure 1) in human ovarian carcinoma.

Materials and methods

Chemicals and reagents

3-cinnamoyl-4-hydroxy-6-methyl-2H-pyran-2-one (CHP) was purchased from Sigma-Aldrich (St. Louis, MO, USA) and dissolved in dimethyl sulfoxide (DMSO) to a concentration of 100 µM as a stock solution and stored at -20°C. Dulbecco's modified Eagle's medium (DMEM), [3-(4,5-dimethylthiazol-2-yl) -2,5-diphenyltetrazolium bromide] (MTT), trypsin, Hoechst 33258, rhodamine 123, penicillin and streptomycin were purchased from Sigma (Beijing, China). Annexin V-FITC Apoptosis Detection Kit, Cell Cycle and Apoptosis Analysis Kit, Reactive Oxygen Species Assay Kit and BCA Protein Assay kit were purchased from Beyotime Institute of Biotechnology (Shanghai, China). Rabbit antihuman Caspase-3, Bax, Cyclin B1, Cytochrome c, mouse antihuman Bcl-2, and β-actin were purchased from Cell Signaling (China).

Cell lines

OVCAR-3 and OVCAR-420 human ovarian carcinoma cells were purchased from the Cell Bank of Type Culture Collection of the Chinese Academy of Sciences (Shanghai, China). The cells were maintained in media consisting of DMEM, 10% fetal bovine serum, 200 mM glutamine and antibiotics.

MTT assay

2 × 10^5 human ovarian carcinoma cells were adjusted per 100 µl and distributed onto 96-well plates. After 12 h of incubation at 37°C under 5% CO₂ atmosphere, the cells were treated with different concentrations of CHP for 72 h. To each well 20 µl of MTT (dissolved 5 mg/ml) was added. The incubation for 4 h was followed by addition 150 µl of DMSO to each plate. The plates were placed on an orbital shaker for 5 min. EnSire™ 2300 Multilabel Plate Reader (PerkinElmer, Inc., Waltham, MA, USA) was used to measure the absorbance at 595 nm.

Annexin V/propidium iodide (PI) double staining

Human ovarian carcinoma cells were treated with different concentrations of CHP for 72 h and then washed with phosphate-buffered saline (PBS). The cells after suspending in binding buffer [10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (pH 7.5), 2.5 mM CaCl₂ and 140 mM NaCl] were incubated with Annexin V-fluorescein isothiocyanate and PI for 15 min in the dark. FACSCalibur™ flow cytometer (Becton, Dickinson and Company, Franklin Lakes, NJ, USA) was used for detection of apoptotic cells. For quantifying apoptotic cells we used Annexin V-fluorescein isothiocyanate (FITC)/PI kit (BD Biosciences, San Jose, CA, USA) and analysis was performed using Modfit and Cell Quest™ software (Becton, Dickinson and Company) were used. Annexin V-positive cells were considered to be in the early stage of apoptosis, whereas Annexin V and PI-positive cells were considered to be in the late stage of apoptosis.

Flow cytometric analysis of cell cycle

The cell cycle phase distribution in OVCAR-3 human ovarian carcinoma cells was determined by using PI and flow cytometry as reported earlier [24]. Briefly, the cells were treated with CHP, incubated for 72 h and then fixed in 70% ice-cold ethanol overnight. The cells washed twice with PBS were stained for 1 h with 50 µg/ml PI (containing 100 µg/ml RNase A) at 4°C in dark. Beckman Coulter, Epics XL (USA) using Cell Quest software was used for DNA contents assay.

Determination of intracellular reactive oxygen species (ROS) generation

Generation of intracellular ROS was performed by using 2,7-dichlorofluorescein-diacetate (DCFH-DA) staining method. The OVCAR-3
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human ovarian carcinoma cells were cultured in 6-well plates for determination of intracellular ROS generation. The cells were treated with CHP for 72 h and then stained with 10 µmol/L

Figure 2. Effects of CHP on cell cytotoxicity and morphological characteristics of OVCAR-3 and OVCAR-420 cells. (A) OVCAR-3 and (B) OVCAR-420 cells were treated with indicated concentrations of CHP for 72 h. Cell cytotoxicity rates were measured using MTT assay. Data are expressed as Mean ± SE of three independent experiments from similar results. Morphological changes in (C) OVCAR-3 cells and (D) OVCAR-420 cells were observed under the light microscopy after treatment with indicated concentrations of CHP or DMSO (negative control) for 72 h.

Figure 3. Effect of CHP on apoptosis in OVCAR-3 and OVCAR-420 Cells. (A) OVCAR-3 cells were treated with 0, 10, 30 and 50 µM CHP for 72 h. Nuclear morphological changes were observed using Hoechst 33258 staining and fluorescence microscope. Arrows indicate the condensed and fragmented nuclei. Histograms show number of cleaved nuclei (apoptotic cells) counted microscopically from 100 nuclei. (B) OVCAR-3 cells were treated with 0, 10, 20, 30, and 50 µM and (C) OVCAR-420 cells with 0, 10, 20, 30, 40, 50 and 100 µM CHP for 72 h. The cells were then stained with FITC-conjugated Annexin V and PI for flow cytometric analysis. Data are expressed as Mean ± SE of three independent experiments with the similar results.
DCFH-DA at 37°C for 30 min according to manufacturer’s instructions. After staining, cells were washed with PBS and suspended in 200 μl PBS. 2’,7’-dichlorofluorescein (DCF) fluorescence data were acquired on Beckman Coulter, Epics XL (USA).

Western blot analysis

The OVCAR-3 cells were incubated with different concentrations of CHP for 72 h and washed twice with PBS. The cells were treated with lysis buffer and the lysate was centrifuged for 20 min. Bicinchoninic acid (BCA) Protein Assay Kit was used to determine the protein concentration in supernatants. After SDS-PAGE proteins were transferred to polyvinylidene fluoride (PVDF) membranes (Bio-Rad Laboratories, Inc, USA). The PVDF membranes were blocked with 5% (w/v) nonfat milk for 2 h at room temperature followed by washing with Tris-buffered saline-Tween solution (TBST). The membranes were incubated with protein-specific antibody or anti-β-actin antibody (loading control) or cyclin dependent kinase 1 (CDK1) (1:300), respectively at 4°C overnight. Finally, the PVDF membranes were incubated with horseradish peroxidase (HRP) conjugated secondary antibodies and signals were detected using ECL Plus Chemiluminescence Kit on X-ray film (Millipore Corporation, Billerica, USA). For cytochrome c expression in cytosol, cytosolic fractions were extracted as described previously [24].

DNA fragmentation analysis by Hoechst 33258 staining

OVCAR-3 cells were treated with different concentrations of CHP for 72 h, centrifuged at 1000 rpm for 10 min and washed with PBS. The cells were then stained with Hoechst 33258 (50 μg/mL) and incubated at 37°C for 30 min in the dark. The cells were washed and resuspended in PBS and examined under fluorescence microscope (magnification, × 400) (Nikon, Tokyo, Japan) and analyzed using DP2-BSW software. The apoptotic cells showed shrinkage and condensation of chromatin.

Mitochondrial depolarization assay

The changes in mitochondrial membrane potential (ΔΨm) were determined by Rhodamine 123 staining method as reported earlier [25]. In brief, OVCAR-3 cells cultured in 6-well plates were treated with different concentrations of CHP for 24 h. The cells were then suspended in 500 μl PBS and stained with 10 μg/ml rhoda-
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mine 123 at 37°C for 30 min in darkroom. After staining cells were resuspended in 200 μl PBS and Rhodamine 123 fluorescence was measured by flow cytometry.

Statistical analysis

The data presented are the mean of ± S.D. and the results were compared by Student’s t-test. Differences were considered significant at P < 0.05.

Results

CHP inhibits cell proliferation in ovarian cancer cells

To study the effect of a range of CHP concentrations on cell proliferation in human ovarian carcinoma cells we used MTT assay. The results revealed a significant and dose-dependent inhibition of cell growth after 72 h. Among the range of concentrations from 10 to 50 μM tested, a significant inhibitory effect on cell viability was found at 30 and 50 μM concentration of CHP for OVCAR-3 and OVCAR-420 cells, respectively. The maximum cell inhibition in OVCAR-3 and OVCAR-420 cell cultures was 87% and 74%, respectively after 72 h (Figure 2A, 2B). CHP was found to exert a strong inhibitory effect on the viability of OVCAR-3 and SKOV-3R cells, which may contribute to its antitumor potency.

In this study we also found significant changes in cell morphology on CHP exposure. Compared to normal polyclonal structure of cells treated...
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with only DMSO, the CHP treated OVCAR-3 and OVCAR-420 cells acquired a round and shrunken shape. The changes in cell morphology were observable on treatment with 20 μM of CHP. However complete detachment and significant cell death resulted after 72 h treatment with CHP at 30 and 50 μM (Figure 2C, 2D). Therefore, CHP significantly inhibits the growth of ovarian carcinoma cells after 72 h of treatment.

CHP induces apoptosis in ovarian cancer cells

Hoechst 33258 staining analysis was used for DNA fragmentation analysis. The examination showed that 10, 20 and 30 μM CHP significantly increased the percentage of cleaved nuclei to 24.65%, 29.86%, and 63.09% compared to 5.01% (in DMSO control group) in OVCAR-3 cells (Figure 3A). Apoptotic cell death in OVCAR-3 and OVCAR-420 cells was detected by using annexin V/PI double staining followed by flow cytometric examination. We observed that rate of apoptosis depended on concentration of CHP. At 10 μM concentration of CHP, the percentage of OVCAR-3 and OVCAR-420 cells that showed annexin V-FITC binding was 18 and 13% respectively (Figure 3B, 3C). However, treatment of OVCAR-3 and OVCAR-420 cells with 30 and 50 μM concentration of CHP led to annexin V-FITC bound cell percentage of 59 and 52% respectively after 72 h (P < 0.05, at all concentrations). Thus, CHP significantly induces apoptosis in ovarian carcinoma cells.

CHP induces mitotic arrest in human OVCAR-3 cells

Inhibition of cell proliferation is caused by cell cycle arrest. We used PI staining and flow cytometry to investigate the effect of CHP on cell cycle arrest. Exposure of OVCAR-3 cells to increasing concentrations of CHP increased the percentage of cells in G2/M phase with a corresponding decrease in G0/G1 phase. In

Figure 6. Mitochondrial membrane potential in OVCAR-3 cells treated with CHP. Cells were treated with 10 and 30 μM of CHP for 4 h, labelled with Rhodamine 123 and analysed by flow cytometry. The reduction of transmembrane potential after 4 h of treatment is represented as a shift of the fluorescence peak to lower levels and the percentage of cells in the lower fluorescence category was plotted in the graphs.
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OVCAR-3 cell culture, the percentage of cells in G2/M phase increased to 29.67 ± 1.2354% and 56.87 ± 2.0920% after treatment with 10 and 30 μM CHP compared to 16.56 ± 1.0231% in DMSO control group (Figure 4A, 4B).

**CHP induces increased generation of ROS**

Reactive oxygen species assay kit was used to measure ROS generation in OVCAR-3 cells. The results showed increase in levels of ROS to 14.24 ± 0.9132% and 27.82 ± 1.8665% respectively after treatment with 10 and 30 μM CHP (P < 0.01), compared to 6.21 ± 0.7668% in DMSO control group (Figure 5). Thus, CHP treatment leads to increase in ROS generation in OVCAR-3 cells.

**CHP disrupts mitochondrial membrane potential (MMP)**

As CHP increases generation of ROS, which is associated with lipid peroxidation, protein oxidation and DNA damage. These changes lead to alteration of mitochondrial membrane potential. We used Rho-123 staining and flow cytometry assay to determine MMP in OVCAR-3 cells. The results revealed that CHP treatment significantly decreased MMP to 85.87 ± 1.2443% & 57.52 ± 1.6554% at 10 and 30 μM respectively in OVCAR-3 compared to 92.71 ± 2.1243% in control group (Figure 6).

**Effect of CHP on major cell cycle regulators and mitochondrial apoptosis regulatory proteins**

The results from Western blot analysis clearly indicated that CHP treatment increases the expression of pro-apoptotic proteins Caspase-3, Bax, and release of cytochrome c (Figure 7A). However, there was a significant decrease in anti-apoptotic protein, Bcl-2, CDK1 and cyclin B1 expression on treatment with CHP at a concentration of 20 and 30 μM in OVCAR-3 cells (Figure 7B).

**Discussion**

Among available cancer treatment therapies, chemotherapy is the most important therapy for ovary carcinoma. Although cisplatin has been widely used for decades to treat a wide range of cancers but development of resistance poses a major clinical challenge. Coumarins [11, 12] have been reported to exhibit potent anticancer activity by inducing apoptosis due to activation of mitochondrial pathway and caspase-3-dependent apoptotic pathway [13, 14]. Similarly, their derivative like 7,8-Dihydroxycoumarin and its analogs, psoralidin [20] xanthoxyletin [21] and RKS262 [22] also exhibit promising anticancer potential.

The present study demonstrates that CHP inhibits growth of human OVCAR-3 and OVCAR-420 cells which might be due to cell cycle arrest and cell death. The CHP treated OVCAR-3 and OVCAR-420 cells acquired a round and shrunken shape in comparison to normal polyclonal structure of cells treated with only DMSO. In order to evaluate the cause of cell cycle arrest and cell death, we used flow cytometry analysis. The results revealed that CHP induced cell cycle arrest at G2/M phase of the cell cycle. Regulation of cell transition from G2 to M phase is controlled by different combinations of cyclins and the cyclin-dependent kinases
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(CDKs) [26-28]. Western blot analysis showed a decrease in expression of both Cyclin B1 and CDK1 in OVCAR-3 cells on treatment with CHP. Therefore, the cell cycle arrest at G2 phase might be due to CHP-induced decrease in expression of Cyclin B1 and CDK1. The cytotoxic agents are reported to arrest the cell cycle at specific phase leading to apoptosis [29-32]. The cytometric analysis of OVCAR-3 and OVCAR-420 cells showed that CHP induced apoptosis in concentration dependent manner.

Increased ROS generation leads to mitochondrial apoptosis [24]. We used reactive oxygen species assay kit to monitor effect of CHP on ROS generation and found significant increase in generation of ROS. It is known that increased generation of ROS induces depolarization of mitochondrial membrane potential. The results from flow cytometric analysis of CHP-treated OVCAR-3 cells showed that MMP was significantly decreased compared to only DMSO treated control group. The effect of CHP on expression of Bcl-2 and Bax proteins was studied using Western blot analysis. There was decrease in expression of anti-apoptotic Bcl-2, CDK1 and cyclin B1 proteins on treatment with CHP. On the other hand expression of pro-apoptotic Bax protein increased. CHP treatment was also found to promote release of Cytochrome c into cell cytosol. All these observations led to a conclusion that CHP induces apoptosis through intrinsic pathway.

In summary, the results of our study demonstrate for the first time that CHP induces apoptosis in OVCAR-3 and OVCAR-420 cells. The CHP-mediated apoptosis is caused by excessive ROS generation, increase in expression of pro-apoptotic and decrease in expression of anti-apoptotic proteins, changes in MMP and Caspase-3 activation. Therefore, CHP may become potential candidate for the treatment of human ovarian carcinoma.

Disclosure of conflict of interest

None.

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