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Hinokiflavone induces apoptosis in melanoma cells through the ROS-mitochondrial apoptotic pathway and impairs cell migration and invasion

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ABSTRACT

Melanoma, the highest degree of malignancy, is one of the most common skin tumors. However, there is no effective strategy to treat melanoma in current clinical practice. Therefore, it is urgent to find an efficient drug to overcome melanoma. Here, the in vitro anticancer effects of a natural product named hinokiflavone on three melanoma carcinoma cell lines (human melanoma A375 and CHL-1 cells, murine melanoma B16-F10 cells) and mechanisms of action were explored. The results of MTT assay revealed that hinokiflavone inhibited cell proliferation of these cell lines in a dose- and time-dependent manner. Interestingly, hinokiflavone showed low toxicity to normal liver cells. Flow cytometry assay and EdU incorporation assay indicated that hinokiflavone affected A375 and B16 cells survival by inducing apoptosis and blocking cell cycle progression at S phase in a concentration-dependent manner. Moreover, hinokiflavone enhanced the reactive oxygen species (ROS) and decreased the mitochondrial membrane potential obviously. Furthermore, hinokiflavone effectively impaired A375 cells migration and invasion, and down-regulated the expression of matrix metalloproteinase (MMP) MMP2 and MMP9. The above-mentioned results demonstrated that hinokiflavone could be a novel chemotherapeutic agent in melanoma treatment by inhibiting cell proliferation, inducing apoptosis and cell cycle arresting and blocking cell migration and invasion.

1. Introduction

Although melanoma represents a small subset, it is the most deadly cutaneous neoplasm with an increasingly common malignancy affecting a younger population than most cancers [1]. Now melanoma has become a major public health problem in many countries, especially in the western countries which have a larger population of white race. The high incidence and mortality rates of melanoma are continuous increasing around the world [2]. Based on the recent statistics analysis on both male and female, the melanoma is the third most common tumor in Australia. Simultaneously, it is ranked fifth in both men and women in American where the skin-cancer is the most common malignancy [3]. Approximately 4.6% of new cases accounting for 73,870 melanoma patients were diagnosed in United States in 2014 [4].

It is gratifying that the detection and treatment of melanoma have been notable developed in recent years, especially in Western Europe, Australia and North America [5]. Currently, the primary modes of melanoma treatment are surgery, adjuvant chemotherapy (including the Vemurafenib, dabrafenib, trametinib dacarbazine etc.) [6,7], passive immunotherapy, radiation therapy and vitamin therapy [8]. However, the above conventional therapeutic strategies applied in clinical treatments were unsatisfactory and the prognosis was also insufficient due to the high metastatic potential and ubiquitous drug resistance [9–11]. Therefore, it is challenging and urgent to find novel anti-melanoma candidates [12].

Abbreviations: MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; DMSO, dimethyl sulfoxide; PBS, phosphate-buffered saline; FBS, fetal bovine serum; FCM, flow cytometry; ROS, reactive oxygen species; DCFH-DA, 2',7'-dichlorodihydrofluorescein diacetate; Rh123, rhodamine123; PI, propidine iodide; WB, western blot; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; PVDF, polyvinylidene difluoride; MMP2, matrix metalloproteinase-2; MMP9, matrix metalloproteinase-9; S.D., standard deviation

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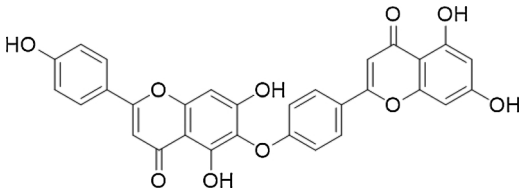

Fig. 1. Chemical structure of Hinokiflavone.

Since more and more relevant evidences manifested that natural products relating closely to human life had crucial anti-tumor activities, the natural products and the derivatives are becoming ones of the foremost approaches to obtain anti-neoplastic drugs for human [13,14]. It was reported at least 75% of anti-tumor drugs are derived from or inspired by natural products, such as paclitaxel and colchicine [15]. More importantly, as one of the most momentous sources of drug lead, the natural products illustrated their intrinsically unique structures [16].

Hinokiflavone (Fig. 1), a natural product, isolated from several plants such as Selaginella P. Beauv, *Juniperus phoenicea* and *Rhus succedanea* and so on [17–19], has been reported with medicinal function in several works, including anti-HIV-1 reverse transcripatase [20], anti-influenza virus sialic acid enzyme [21] and antioxidant activity [22]. Lin et al. reported hinokiflavone possessed antitumor activity in KB human oral cancer cells in vitro [23]. Sukesh Kalva et al. identified that hinokiflavone had a good inhibitory activity against MMP2 and MMP9 [18]. MMP2 and MMP9, affiliated to the matrix metalloproteinase (MMP) family, were demonstrated as attractive targets for anticancer therapy, which was involved in the tumor invasion, metastasis, growth and neovascularization [24–26]. Meanwhile, several literatures attested the ability in metastasize of melanoma was closely related to the MMPs [27,28].

This study evaluated the anticancer effects of the hinokiflavone on three melanoma carcinoma cell lines and explored the mechanisms of action in vitro. Our results indicated that the hinokiflavone inhibited cell proliferation, induced apoptosis and cell cycle arrest at S phase, and suppressed migration and invasion in melanoma cell lines. These data suggest that the hinokiflavone is a potential novel drug candidate to treat melanoma.

2. Materials and methods

2.1. Reagents

Hinokiflavone as a 98% purity that measured via HPLC was obtained from Chengdu Biopurify Phytochemicals Ltd (Chengdu, China). 3-(4,5-Dimethylthiazol-2-yl)-2,5- diphenyltetrazoliumbromide (MTT), 2′,7′-dichlorofluorescein diacetate (DCFH-DA) and 2-(6-Amino-3-imino-3H-xant-hen-9-yl) benzoic acid methyl ester (Rh123) were purchased from Sigma Chemical Co. (St Louis, MO, U.S.A.). The Annexin V-FITC and PI Apoptosis Detection Kit were purchased from KeyGen Biotech (Nanjing, China). Hoechst33258 was obtained from KeyGen Biotech. The primary antibodies against Bax, Bcl-2, caspase-3, cleaved-caspase-3, β-actin, matrix metalloproteinase-2 (MMP2) and matrix metalloproteinase-9 (MMP9) were purchased from Cell Signaling Technology (Beverly, MA, U.S.A.) for western blot experiments. Secondary antibodies were supplied by ZSGB-BIO Co. (Beijing, China).

Hinokiflavone (40 mM) dissolved in DMSO was stored at −20 °C. The stock solution was diluted with culture medium for further application.

2.2. Cell culture

The human malignant melanoma A375 cells and CHL-1 cells, mouse malignant melanoma B16 cells (B16-F10 cells), normal human hepatocytes cells LO2, and normal African green monkey kidney cells Vero were purchased from American Type Culture Collection (ATCC, Rockville, MD, USA). Dulbecco's Modified eagle medium (DMEM) containing 1% antibiotics (penicillin and streptomycin) and 10% heat-inactivated fetal bovine serum (FBS, Gibco, Auckland, N.Z.) was applied. Cell was incubated with 5% CO_2 and 37 °C.

2.3. Cell proliferation assay

Cells (2-5 × 10^3/100 μL/well) were seeded in the 96-well plates and treated with 0, 0.625, 1.25, 2.5, 5, 10, 20 and 40 μM hinokiflavone after 24 h. After incubation of 24, 48 and 72 h, 20 μL of MTT solution (5 mg/mL) was added to each well. The cells were continuously incubated for 4 h. The purple formazan crystal was dissolved in 150 μL of DMSO and subsequently recorded the absorbance at 570 nm using a Spectra MAX M5 microplate spectrophotometer (Molecular Devices, CA, U.S.A.).

2.4. Colony forming efficiency

As the previous literature depicted [29], colony formation assay was measured. In short, A375, B16 and CHL-1 cells were seeded in six-well plates at 200–500 cells/well respectively. After 24 h incubation, the cells were treated with various concentrations (0, 1.25, 2.5, 5, 10, and 20 μM) of hinokiflavone for 9 days. Then the cells were fixed with methanol and stained with 0.5% crystal violet solution for 15 min. The colonies (> 50 cells) were counted under the microscope. Data shown represent the average of three independent experiments.

2.5. Morphological analysis by Hoechst33258 staining

As the article described [29], morphologic features, including cell shrinkage and the formation of apoptotic bodies, were utilized to identify the cell apoptosis. In the staining test, A375 cells and B16 cells owning a 50% and 30% consistency were plated onto 18-mm coverslips in a six-well plate for 24 h respectively. After 24 h treatment with diverse concentrations (0–40 μM) of hinokiflavone, the cells were washed with ice-cold phosphate-buffered saline (PBS) twice and fixed in ice-cold methanol for around 15 min. The cells stained with the Hoechst33258 solutions were finally photographed by a fluorescence microscopy (Leica, DM4000B) in order to observe the nuclear morphology in particular occurring apoptotic bodies.

2.6. Apoptosis analysis by flow cytometry (FCM)

Further verification on apoptosis induced by hinokiflavone was carried out by AnnexinV-FITC/PI apoptosis detection kit. After incubation with various concentrations of hinokiflavone (0–40 μM) for 24 h, the cells were collected and washed twice with ice-cold PBS. The apoptotic cells were analyzed via flow cytometry (FCM, BD Bioscience). FlowJo software was applied to analyze the data to present the average of three independent experiments.

2.7. Cell cycle assay by flow cytometry (FCM) and the EdU incorporation assay

A375 cells and B16 cells treated with hinokiflavone for 24 h were fixed in 75% ethanol and incubated with 500 μL PBS containing 50 μg/mL PI and 0.1% Triton X-100 in the dark. The samples were analyzed by FCM to obtain the condition of the cycle distribution on A375 cells and B16 cells.

EdU incorporation assay was used to label proliferating cells on account of incorporating into replicating DNA in S stage. In short, A375 cells and B16 cells were seeded in 96-well plates (5000 cells/100 μL/well) and treated with a series of concentrations of hinokiflavone for 24 h. Following, we used the Cell-Light™ EdU DNA Cell Proliferation Kit to analyze the cell proliferation based on the manufacturer's