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The antitumor effect of formosanin C on HepG2 cell as revealed by ^{1}H-NMR based metabolic profiling

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ABSTRACT

Formosanin C (FC) is a pure compound isolated from Rhizoma Paridis. In the past years, antitumor effects of FC have been observed in several cultural cells and animal systems. However, there was no research particular on liver cancer. In this experiment, 3-(4, 5-dimethylthiazol diphenyltetrazolium bromide (MTT) dye reduction assay was used to evaluate cell viability of HepG2 cells with FC-treatment. 4′, 6-diamidino-2-phenylindole (DAPI) staining, Annexin V-FITC/PI assay and DNA fragment assay were applied to observe FC-induced apoptosis. Cell cycle analysis and NMR metabolic profiles were used to identify molecular mechanisms of FC in HepG2 cells. As a result, FC inhibited the growth of HepG2 cells through inducing apoptosis and S phase arrest. Cells cultured in the presence or absence of FC was different in metabolic profiles. The treatment decreased acetate, ethanol, choline and betaine, and increased butyrate, fatty acids, leucine and valine in HepG2 cells. In conclusion, metabolomic analysis of the exo-metabolome of FC-treated HepG2 cells, together with traditional methods such as apoptosis test and cell cycle analysis provided a holistic method for elucidating mechanisms of potential anti-cancer drug, FC.

1. Introduction

Hepatocellular carcinoma (HCC) is the fifth most common type of carcinoma all over the world, and accounts for about 6% of all new cancer cases diagnosed worldwide (nearly 750,000 new cases/year) [1]. It is an urgent need to develop new therapeutic agents to fight against liver cancer.

Formosanin C (FC), a diosgenin glycoside with four sugars, has only recently emerged as a potential antitumor agent [2–4]. It was an effectively promoting agent for cell cycle arrest and apoptosis without deleterious effects to different normal cell types or benign neoplastic derived cells [3]. Up to now, FC appeared to be sensitive to many kinds of cancer cells including myeloid leukemia, colon cancer, liver cancer, lung cancer, cervical cancer and renal adenocarcinoma cells [5].

The aim of the present paper was to investigate the anti-cancer effects of FC on human liver cancer cell lines HepG2 and the possible mechanisms based on metabonomics.

2. Materials and methods

2.1. Materials

3-(4,5-Dimethyl-thiagol-2yl)-2,5-diplenyltertrazollium (MTT) and 4′,6-diamidino-2-phenylindole (DAPI) were obtained from Solarbio Science & Technology Co., Ltd. (Beijing, China). Apoptotic DNA ladder isolation kit and cell cycle analysis kit were purchased from Beyotime Institute of Biotechnology (Jiangsu, China). High-glucose DMEM medium, fetal calf serum, penicillin–streptomycin, trypsin and EDTA were obtained from Thermo (Being, China). Sodium-3-(trimethylsilyl)-2,2,3,3-tetradeuteriopropionate (TSP) was purchased from Merck (Germany). Deuterium oxide (D_2O) was purchased from J&K, China.

2.2. Isolation of FC

Paris polyphylla Smith var. yunnanensis was collected from Lijiang, Yunnan province of China. The plants were air-dried,

Abbreviations: DAPI, 4′,6-diamidino-2-phenylindole; D_2O, deuterium oxide; FC, formosanin C; HCC, hepatocellular carcinoma; MTT, 3-(4,5-dimethyl-thiagol-2yl)-2,5-diplenyltertrazollium; TSP, sodium-3-(trimethylsilyl)-2,2,3,3-tetradeuteriopropionate.

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chipped and extracted with ethanol and partitioned with petroleum ether, EtOAc and n-BuOH, sequently. The n-BuOH soluble fraction was separated on a silica gel column with CHCl3:MeOH (100:0 → 95:5 → 9:1 → 8:2 → 7:3 → 6:4 → 0:10), a Sephadex LH20 column with CHCl3: MeOH (1:1) and an HPLC-ODS with MeOH → MeOH:H2O (75:25) to obtain FC [6]. Reference standard of FC was purchased from the National Institute for the Control of Pharmaceutical and Biological Products, China. Its batch was 111,591–200,402. The purity of this monomer was determined to be more than 98% by normalization of the peak areas detected by HPLC, and was stable in methanol solution [7].

2.3. Cell culture

Human hepatoma cell line HepG2 was acquired from Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences (Shanghai, China). The HepG2 cells were maintained in high-glucose DMEM supplemented with 10% heat-inactivated (56 °C, 30 min) fetal calf serum, penicillin (100 U/mL) and streptomycin (100 μg/mL). The cell culture was maintained at 37 °C in a humidified atmosphere of 5% of CO_2 with passage each 3–4 days.

2.4. Cell viability assay

The cell viability assay was evaluated by MTT assay [8]. HepG2 cells were seeded in 96-well microtiter plates at a density of 1×10^4 per well and left to adhere overnight before drugs treatment. FC was prepared in the basal medium with a final DMSO concentration of less than 0.1%. The same concentration of DMSO was used as the vehicle control in all experiments. The cells were then incubated in the presence of 0 to 50 μg/mL of FC for 24 and 48 h. MTT was added to each well at a concentration of 0.5 mg/mL and incubated for 4 h. Absorbance was determined at 570 nm by addition of 100 μL of DMSO for each well using ELISA reader. The experiments were repeated in triplicate wells.

2.5. DAPI staining

Approximately 2×10^5 cells/well of HepG2 cells seeded in 6-well plate and left to adhere overnight. Then different concentrations of FC (5 and 10 μg/mL) were added in the cells and incubated for 24 or 48 h. Cells were stained by DAPI (5 μg/mL) for 10 min at room temperature, and then the DAPI dye was aspirated. The DAPI staining cells were photographed by fluorescence microscope.

2.6. DNA fragmentation

Apoptosis was monitored by DNA fragmentation assay. Approximately 1×10^6 cells/well of HepG2 cells seeded in 6-well plate and left to adhere overnight. Then different concentrations of FC were added in the cells and incubated for 48 h. Later DNA fragmentation was extracted by Apoptotic DNA ladder isolation kit. Fragmented DNA was electrophoresed on 1.5% agarose gel to observe the appearance of DNA ladder.

2.7. Annexin V-FITC/PI staining for apoptosis evaluation

Apoptosis was quantified using flow cytometry to measure the levels of detectable phosphatidylserine on the outer membrane of apoptotic cells. HepG2 cells were seeded on 6-well plates (4×10^5 cells/mL), and incubated with different concentrations of FC in 0.1% DMSO solutions for 48 h. Then the cells were harvested by trypsinized, washed in ice-cold phosphate buffered saline, and re-suspended in diluted binding buffer from the Annexin V-FITC kit (Bestbio Inc., Shanghai, China) based on the manufacturer's instructions. The procedure was carried out three times.

2.8. Cell cycle analysis

The cell cycle distribution was analyzed by cell cycle analysis kit (Beyotime, China) according to the manufacturer's instruction. Briefly, HepG2 cells were treated with different concentrations of FC for 24 h. Then the cells were harvested by trypsinized, washed in ice-cold phosphate buffered saline, and fixed in 70% ice cold ethanol overnight. Subsequently, the fixed cells washed with ice-cold PBS before incubation with the binding buffer containing RNase and propidium iodide for 30 min at 37 °C in the dark. Finally, the stained cells were analyzed by flow cytometry with modfit LT software (Modfit LT 4.0).

2.9. ^{1}H-NMR analysis

Approximately 3×10^6 cells/well of HepG2 cells seeded in cell culture dishes and left to adhere overnight. Then different concentrations of FC were added in the cells and incubated for 24 h. Collecting cells and the change of the metabolites in the HepG2 cells was measured by ^{1}H-NMR [9]. Each sample was mixed with 350 μL of aqueous phosphate solution (0.2 mol/L). The addition of an internal standard like TSP (100 μL, 1.5 mmol/L) was prohibited by its interaction with proteins present in the sample. Subsequently, the total volume was transferred to a 5 mm NMR tube.

^{1}H-NMR spectra were measured at a ^{1}H frequency of 400 MHz using a Varian Unity INOVA 600 spectrometer, equipped with a 5-mm triple-resonance probe. Prior to Fourier transformation, the free induction decays were zero-filled to 32 K and an exponential weighing factor corresponding to a line broadening of 0.5 Hz was applied.

3. Results

3.1. FC inhibited viability and proliferation of HepG2 cells

MTT assay showed that FC inhibited HepG2 cell growth in a concentration- and time-dependent manner (Fig. 1). 50% inhibitory concentration (IC50) of FC was estimated to be 13.62 ± 0.36 and 3.29 ± 0.55 μg/mL at 24 and 48 h, respectively. The IC50 of FC was the same as that in another human hepatoma cell line Bel7402 cell.

3.2. Morphological changes caused by FC

It was detected by DAPI staining method that FC inducted HepG2 cells apoptosis after treatment for 24 and 48 h. As shown

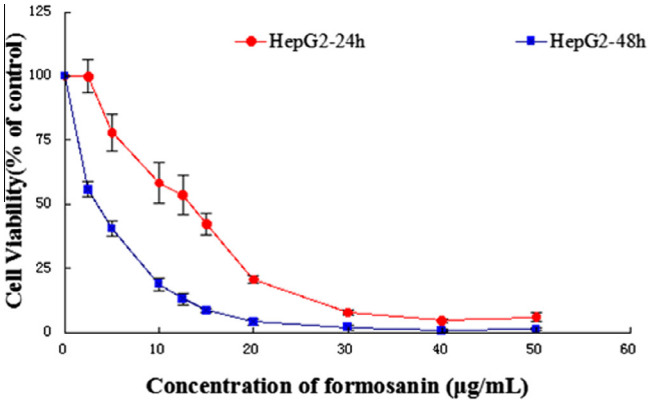

Fig. 1. HepG2 was treated with different concentration of FC for 24 and 48 h. Cell growth was determined by MTT assay and was directly proportional to the absorbance at a wavelength of 570 nm. Data expressed as means ± S.D. from three independent experiments.