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Apoptosis-inducing effects and growth inhibitory of a novel chalcone, in human hepatic cancer cells and lung cancer cells

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ABSTRACT

Apoptosis is an important biological phenomenon, which affects many diseases, such as cancer and Alzheimer's disease. In the present study, we observed that chalcone 9X, an aromatic ketone, induced apoptosis of human hepatic and lung cancer cells and inhibited cancer cell migration and invasion. This compound strongly suppressed the growth of tumor in a mouse model of xenograft tumors. The anticancer activity of chalcone 9X was equivalent to 5-fluorouracil (5-FU) as a positive control agent, whereas the toxic effect of chalcone 9X in non-cancer cells was weaker than 5-FU. Molecular docking results showed that chalcone 9X could act on the active sites of pro-apoptotic proteins capspases-3 and -8 to induce apoptotic death of cancer cells. Our findings suggest that chalcone 9X might be considered a candidate compound of novel anticancer drug in the future.

1. Introduction

Apoptosis is a tightly regulated cell death process which maintains normal cell homeostasis [1]. Abnormal apoptosis has been implicated in a variety of diseases. Some new therapeutic strategies based on apoptosis modulation have been applied to treat diseases, like neurodegenerative diseases [2], cancer [3–5], inflammation [6], Caspase family are important determinants apoptosis. The human caspases are constituted by initiator caspases and executioner caspases. Initiator caspases consist of caspase-2, caspase-8, caspase-9 and caspase-10, among which caspase-8 is the most important one. After dimerization and activation of caspase-8, executioner caspases can be cleaved and activated [7]. Active caspase-8 can also directly induce cell apoptosis [8]. Executioner caspases include caspase-3, caspase-6, and caspase-7. Caspase-3 plays a key role in apoptosis and is an attractive therapeutic target for human diseases associated with apoptosis. The activated or cleaved caspase-3 is considered an important apoptotic marker. Previous works showed that caspase family can be activated by small molecules, such as PAC-1 (procaspase-activating compound 1) [9], compound-1541 [10], compound-42 [11]. Apoptosis in the mitochondria is controlled by Bcl-2 family that includes both anti-apoptotic and pro-apoptotic proteins. Bcl-2, Bcl-xL, Bcl-w and Mcl-1 are anti-apoptotic members, while Bad, Bak, Bax, Bid, Bim and Bcl-xS are pro-apoptotic members [12]. Research shows that mature caspase-8 can cleave Bid into tBid, and promote activation of Bax and Bak. The oligomerization of Bax and Bak induces mitochondrial apoptosis. Bcl-2 protein, inhibitors of Bax and Bak, is negatively regulated by caspase-8 [13].

A number of natural and synthetic chalcones have been reported to possess a variety of bioactivities, such as anti-inflammatory [14], anti-myocardial ischemia [15], immunomodulation [16] and antimalaria [17]. The anticancer efficacy is one of the most important biological activities of chalcones, among which synthetized chalcone, 4'-hydroxyl-3,4-dimethoxyl-3'-methoxylchalcone (chalcone 9X) has been documented to inhibit the growth of human osteosarcoma cells through G2/M cell cycle arrest and apoptosis [18] and induce apoptosis through caspase-dependent intrinsic pathways in human hepatocellular carcinoma cells [19]. We were interested in expanding and deepening our understanding of the mode of actions of chalcones by focusing on chalcone 9X and of the cellular/molecular mechanisms by which chalcone 9X elicits its anti-cancer activity. To this end, we studied the efficacy of chalcone 9X in suppressing tumor growth in a mouse model of xenograft tumors, in inducing apoptosis of liver and lung cancer cells, and in mitigating invasion and migration of cancer cells as well. We also explore the possible direct interaction between chalcone 9X and caspases-3 and -8 using a molecular docking approach.

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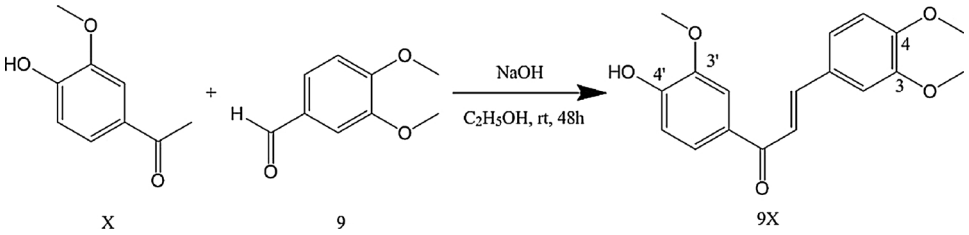

Scheme 1. Synthesis of chalcone 9X.

2. Materials and methods

2.1. General procedures

The purity of the compounds was confirmed by thin layer chromatography using silica gel-GF254. Melting points were taken in open capillary tubes and are uncorrected. IR spectra was recorded on SHIMADZU IR Prestige-21 spectrophotometer in KBr (ν_{max} in cm^{-1}). ^{1}H-NMR spectra were recorded on Bruker AV400MHz spectrometer.

2.2. Chemical synthesis of chalcone 9X

Chalcone 9X was synthesized according to standard Claisen-Schmidt aldol condensation protocols as previously published (Scheme. 1) [20,21]. 3,4-dimethoxyl benzaldehyde (834 mg, 5.02 mmol) was added into a stirred solution of 4-hydroxyl-3-methoxylacetophenone (828 mg, 4.99 mmol) in aqueous NaOH solution in ethanol (40 mL). The whole reaction mixture was stirred at room temperature for 48 h, quenched in ice-cold water, and acidized with 18% HCl. Separated product was filtered and the crude product was recrystallized from ethanol.

2.3. Pharmacology

2.3.1. Cell culture

HepG2, H460, HL-7702 and HBE cells (Zhong Qiao Xin Zhou Biotechnology Co., Ltd., Shanghai, China) were cultured in a DMEM medium or in a RPMI 1640 medium containing 10% fetal bovine serum, at the temperature of 37 °C in 5% CO_2. They were incubated with two different concentrations of chalcone 9X (50 and 100 μmol/L) for 24 h. 5-FU (30 μmol/L) was used as a positive control drug.

2.3.2. Cell viability

We measured cell viability using a Cell Counting Kit-8, CCK8 (Dojindo, Kumamoto, Japan). Cells were plated in 24-well plates at 3×10^5 cells/well. Then cells were incubated in 10% CCK8 reagent which was diluted in normal culture medium at 37 °C for color conversion. The percent cell viability was determined 24 h after the treatment of drugs.

2.3.3. TUNEL staining

DNA fragmentation of individual cells was detected by TUNEL with the Cell Death Detection Kit (TUNEL fluorescence FITC kit, Roche, Indianapolis, IN, USA). Cells grown on coverslips were washed with PBS and fixed in 4% paraformaldehyde solution at 4 °C for 1 h. Then cells were permeabilized in a solution containing 0.1% Triton X-100 for 2 min, followed by incubation in freshly prepared TUNEL reaction mixture at 37 °C in the dark for 1 h. The coverslips were then washed with PBS. Then stained cells were examined under a confocal laser scanning microscope (FV300, Olympus, Japan).

2.3.4. Western blotting

Cells were grinded in 200 μL RIPA buffer containing protease and phosphatase Inhibitors. Lysates were centrifuged at 13500 rpm for 20 min. Then the protein concentration in the supernatant was measured by BCA Protein Assay (Beyotime, Shanghai, China). Equal amounts of protein samples (70 μg) were fractionated on a 12% SDS-PAGE gel electrophoresis and transferred onto nitrocellulose membranes. Then the membranes were blocked in the defatted milk (5%) for 2 h and incubated on the shaker at 4 °C overnight with caspase3 (Wanlei, China, wl01992a), cleaved caspase3 (Wanlei, China, wl01992), caspase8 (Wanlei, China, wl03426), cleaved caspase8 (Abcam, Cambridge, MA, USA, ab25901), Bcl-2 (Wanlei, China, wl01556) and Bax (Wanlei, China, wl01637) antibodies. GAPDH (Zhongshanjinqiao, Inc., Beijing, China, TA309157) was used as an internal control. The membranes were incubated with secondary antibody (Invitrogen) at room temperature for 1 h. Protein bands were visualized and quantified by Odyssey Infrared Imaging System (LI-COR, Lincoln, NE, USA).

2.3.5. Cell migration and Invasion

Cells were seeded in six-well plates with complete DMEM medium to analyze wound healing. After 48 h, the cell monolayer was scratched with a plastic pipette tip. Then the cells were rinsed with PBS and cultured with serum-free DMEM for 24 h. The wound closure was observed and photographed under a microscope. For Transwell assay, 8-μm pore size chambers (Corning, NY, USA) were used with an insert coated with Matrigel (BD Bioscience). Twenty-four hours after drug treatment, 1×10^5 cells in 200 μL serum-free medium were added to the upper chamber. The lower chamber was filled with 500 μL 10% FBS DMEM. The cells remaining on the upper surface of the membrane were removed after 24 h of incubation, whereas the cells that had invaded through the membrane were fixed with 100% methanol for 15 min, stained with 0.1% crystal violet for 20 min. The cells were examined and photographed under a phase-contrast microscope (Olympus, Japan).

2.3.6. Animals and establishment of hepatoma model

30 male BALB/c nu mice (SPF level, Shanghai SLAC Laboratory Animal Co., Ltd) were randomized into five groups: control group, positive control group (5-FU, 10 mg/kg), high (1.12 mg/kg), middle (0.56 mg/kg) and low (0.28 mg/kg) dose groups, 6 animals in each group. Then HepG2 tumor cells were injected subcutaneously into nude mice with the number of 5×10^6/ mouse. Twenty-four hours after the inoculation, drugs were administered by tail vein injection once a day for two weeks. Tumor growth was observed and recorded for 20 days.

2.3.7. Statistical analysis

Group data are described as the mean ± S.E.M. One-way ANOVA accompanied by Bonferroni's Multiple Comparison Test was used to analyze comparisons. $p < 0.05$ was considered to indicate a significant difference. Data were analyzed using the GraphPad Prism 5.0 software.

2.4. Molecular docking

The crystal structures of caspase-3 (PDB: 2J30) and caspase-8 (PDB: 3kjn) were downloaded from RCSB Protein Data Bank (http://www.rcsb.org/). The structures of caspase-3 and caspase-8 were prepared with the biopolymer tool of Sybyl-X 2.0 (Tripos, USA). Hydrogen atoms were added and AMBER FF99 charges were calculated for the protein. The water in protein was deleted. A 1000 iteration minimization of the hydrogen atoms was followed by a 100 ps molecular dynamics simulation to refine the positions of targets. Prior to docking, chalcone 9X