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 caspase-3 and -8 to induce apoptotic death of cancer cells.

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 of 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 [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], antimycocardial ischemia [15], immunomodulation [16] and antimalarial [17]. The anticancer efficacy is one of the most important biological activities of chalcones, among which synthetized chalcone, 3,4-dimethoxyl-3'-methoxychalcone (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 caspase-3 and -8 using a molecular docking approach.

References

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 (νmax in cm⁻¹). ¹H-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, Biotechnology Co., Ltd., Shanghai, China) were cultured in a DMEM medium containing 10% fetal bovine serum. Cells grown on coverslips were washed with PBS. Then stained cells were examined under a confocal laser scanning microscope (FV300, Olympus, Japan).

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 × 10⁴ 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 FTTC 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, w01992a), cleaved caspase3 (Wanlei, China, w01992), caspase8 (Wanlei, China, w03426), cleaved caspase8 (Abcam, Cambridge, MA, USA, ab25901), Bcl-2 (Wanlei, China, w01556) and Bax (Wanlei, China, w01637) 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 × 10⁵ 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 × 10⁶/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: 3kjm) 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...
was minimized with the tripods force field. Inputted ligand file format was mol2 for all docking programs investigated.

Chalcone 9X docking with targets were accomplished with Surflex–Dock in the Sybyl-X 2.0. The SFXC file was built using the PDB prepared protein structure. The protocol was generated using the AMPPD ligand with a threshold of 0.50 and bloat set to 0 (default settings). The docking pockets are shown in Fig. 6C–D. Cscore calculations were enabled on Surflex docking runs. Additional starting conformations per molecular were 10. Angstroms to expand search grid were 6. Max conformations per Fragment were 30. Max number of rotatable bonds per molecular was 150. The maximum number of poses per ligand was 50. And the minimum RMSD between final poses was 0.05. Surflex select compound poses with incremental construction algorithms. The number of poses returned by each docking program was determined by the default settings, and the poses were scored using native scoring function [22]. The docking results were analyzed using Sybyl-X2.0.

3. Results

3.1. The compound of chalcone 9X

Chalcone 9X (0.655 g, 41.7%) is a pale yellow solid substance with chemical formula C_{18}H_{18}O_{5} and molecular weight 314. m.p.: IR (KBr, cm^{-1}): 3435 (O-H), 2833 (OCH_{3}), 1653 (C = O), 1585, 1514 (C = C), 1271, 1136, 1020 (ArH), 819 (=CH); 1H-NMR (400 MHz, CDCl_{3}) ppm 7.77 (d, J = 15.6 Hz, 1H, =CH), 7.65 (d, J = 9.7 Hz, 2H ArH), 7.42 (d, J = 15.4 Hz, 1H, =CH), 7.25 (d, J = 11.1 Hz, 2H, ArH), 7.17 (s, 1H, ArH), 7.00 (d, J = 8.0 Hz, 1H, ArH), 6.91 (d, J = 8.2 Hz, 1H, ArH), 6.10 (s, 1H, OH), 4.02–3.91 (t, 9H, OCH_{3}).

3.2. Chalcone 9X promotes apoptosis of HepG2 and H460 cells

CCK8 assay showed that chalcone 9X at concentrations of 50 and 100 μmol/L significantly decreased cell viability of HepG2 and H460 (Fig. 1A, B). Furthermore, TUNEL staining to detect DNA fragmentation as an indication for apoptosis demonstrated that the percentage of HepG2 hepatic cancer cells and H460 lung cancer cells with positive TUNEL staining was markedly increased by chalcone 9X compared with the control group (Fig. 1C–F).

3.3. Toxic effects of chalcone 9X on HL-7702 liver cells and HBE lung cells

To evaluate the toxic effects of chalcone 9X on normal cells, cell viability of HL-7702 non-cancer liver cells and HBE non-cancer lung cells was detected by CCK8 assay. Our results showed that chalcone 9X at 50 μmol/L for 8 h did not affect the cell viability, and it at 100 μmol/L caused a small but statistically significant decrease in viability, similar to 5-FU at 30 μmol/L (Fig. 2A, B). However, after 24 h treatment, chalcone 9X at both 50 and 100 μmol/L resulted in appreciable and statistically significant decreases in cell viability (Fig. 2C, D) in a concentration-dependent manner.
3.4. Chalcone 9X alters expression of apoptosis-related proteins in liver and lung cancer cells

To get insight into the cellular/molecular mechanisms underlying the apoptosis promoting property of chalcone 9X in liver and lung cancer cells, we went on to investigate the effects of this compound on expression of the apoptosis-mediating signaling molecules. As illustrated in Fig. 3A–E and Fig. 4A–E, while the total protein levels of caspase-3 and caspase-8 remained unaltered, the activated forms of these enzymes (cleaved-caspase3 and cleaved-caspase8) were robustly upregulated by two different concentrations of chalcone 9X and 5-FU in both HepG2 and H460 cancer cells. Moreover, anti-apoptotic factor Bcl-2 was significantly decreased by both chalcone 9X and 5-FU (Fig. 3C and Fig. 4C), whereas pro-apoptotic protein Bax was markedly increased (Fig. 3F and Fig. 4F).

3.5. Chalcone 9X inhibits invasion and migration of HepG2 and H460 cells

We then sought to explore whether chalcone 9X also acts on the invasion and migration ability of HepG2 and H460 tumor cells in addition to its pro-apoptotic action. As depicted in Fig. 5A, B, invasion of HepG2 cells was significantly reduced by chalcone 9X in a concentration-dependent fashion and by 5-FU as well, as revealed by transwell assay. Moreover, cell migration was considerably mitigated by chalcone 9X (Fig. 5C, D). Similar results were observed with H460 cells (Fig. 6).

3.6. Chalcone 9X inhibits tumor growth in a mouse model of xenograft tumors

We then subsequently examined the effects of chalcone 9X on tumor growth with BALB/c nu mice model. Three dosages of chalcone 9X (0.28, 0.56 and 1.12 mg/kg) were administered by tail vein injection starting from one day after inoculation of tumor cells to nude mice once a day for consecutive 14 days, and tumor growth was monitored for up to four weeks (Fig. 7A). 5-FU group was used as a positive control compound. Significant differences in tumor size between drug treated mice and vehicle-treated control counterparts were observed starting from day 5 following drug administration (Fig. 7B). All three dosages of chalcone 9X elicited significant suppressive effects against tumor growth, consistent with the action of 5-FU. Moreover, high dose of chalcone 9X was the most effective.
3.7. Chalcone 9X alters expression of apoptosis-related proteins in tumor tissues

Consistent with the data shown above, chalcone 9X significantly increased the protein levels of cleaved caspase-3 (Fig. 8A, D) and cleaved caspase-8 (Fig. 8B, E), as well as Bax (Fig. 8F) in a dose-dependent manner, whereas it diminished the protein level of Bcl-2 (Fig. 8C). These results suggested that chalcone 9X inhibited the tumor growth by promoting cell apoptosis.

3.8. In silico molecular docking analysis

To shed light on the mechanisms by which chalcone 9X affects the activation of caspase-3 and -8, we conducted docking analysis using Surflex-Dock (Tripos, USA) to exploit the possible interactions between chalcone 9X and the two enzymes. The analysis predicts four amino acids (His121, Arg207, Asn208 and Ser209) in caspase-3 protein that could be the potential target site for binding of chalcone 9X by forming H-bond with 4'-hydroxyl, 3'-methoxyl, 3-methoxyl and 4-methoxyl groups of chalcone 9X (Fig. 9A). Similarly, Fig. 9B shows that the carbonyl group of Ser411 and amino group of Arg413 of caspase-8 protein might serve as an acceptor and a donor to form H-bonds with chalcone 9X. Fig. 9C-D shows that chalcone 9X might interact with caspase-3 and caspase-8 in the hydrophobic regions. Specifically, the active region of caspase-3 seemed to lie on the residues His121, Gly122, Gln161, Ala162, Cys163, Tyr204, Ser205, Trp206 and Arg207 (Fig. 9C). 3'-methoxyl groups and 4'-hydroxyl group of chalcone 9X could insert into an active pocket of caspase-3 and form a strong combination with the latter. Similarly, chalcone 9X could combine with the hydrophobic regions of caspase-8 with the amino acid residues Leu315, Ser316, His317, Gln358, Ala359, Cys360, Ser411, Tyr412 and Arg413 that can together form an active pocket (Fig. 9D). 3'-methoxyl groups and 4'-hydroxyl group of chalcone 9X could penetrate into this active pocket.

4. Discussion

5-FU is one of the most important drugs for treating solid cancers. Large amount of evidence has identified the apoptosis promotion effects of 5-FU on different tumors, including colon cancer [23], hepatocellular carcinoma [24] and gastric cancer [25]. Chalcone 9X is an aromatic ketone and an enone that forms the central core for a variety of important biological compounds. In our study, the cell viability of HepG2 and H460 cells treated with 50 μmol/L chalcone 9X was similar to that treated with 30 μmol/L 5-FU. While cell viability of non-cancer liver and lung cells treated with 50 μmol/L chalcone 9X was much higher than that treated with 30 μmol/L 5-FU. These results suggested chalcone 9X has lower toxicity than 5-FU, but has equivalent anti-tumor activity to 5-FU. Our data further unraveled that chalcone 9X promoted cell apoptosis, and this pro-apoptotic property is likely conferred by the ability of this compound to promote activation caspases-3 and -8, and to
downregulate Bcl-2 and upregulate Bax expression.

In addition to its pro-apoptotic action, chalcone 9X was found to be able to suppress invasion and migration of cancer cells with equivalent efficacy to 5-FU. It is therefore likely that chalcone 9X suppressed tumor growth in a mouse model of xenografts by a combination of its pro-apoptosis and anti-invasion/migration effects. These new findings should broaden our understanding of the anti-cancer pharmacology of chalcone 9X.

Our molecular docking results indicate that chalcone 9X could well fit to the active regions of caspase-3 and caspase-8, respectively. The 4’-hydroxyl and 3’-methoxyl groups are believed to be the key pharmacophores which form H-bonds with key amino acid residues in active sites of targets, and 3,4-dimethoxyl groups could serve as acceptors of H-bond or medium hydrophobic fragments. In our model, chalcone 9X appears to have the stronger H-bond binding force with caspase-3 than with caspase-8. His121, Arg207, Asn208 and Ser209 of caspase-3 could simultaneously form H-bond with chalcone 9X. We therefore proposed that chalcone 9X could directly induce conformation changes of caspase-3 protein into mature caspase-3 or cleaved caspase-3. The results in Fig. 9B also suggest that chalcone 9X could also interact with the key amino acid residues of allosteric region and caused conformational changes to form mature caspase-8. Next, mature caspase-8 as an allosteric activator of caspase-3 induced the formation of cleaved caspase-3. Meanwhile, mature caspase-8 could also affect the expression of Bax and Bcl-2. These are consistent with our data showing that chalcone 9X repressed the expression of anti-apoptotic Bcl-2 and raised the level of pro-apoptotic Bax in cancer cells. According to the molecular docking results shown in Table 1, Caspase-3 (PDB: 2J30) has a maximum polar value, indicating the optimal polar interaction between chalcone 9X and target. The crash value reflects the degree of intermolecular collision. Caspase-8 (PDB: 3kjn) has the minimum crash value, indicating that chalcone 9X could fit well with the active site of caspase-8.

5. Conclusions

Our data provided the evidence for the previous hypothesis that chalcone 9X could affect the active sites of two caspases simultaneously to induce apoptosis of HepG2 and H460 cells. Chalcone 9X seemed to have lower toxicity than 5-FU at the concentrations that produced similar anti-cancer efficacies. In addition, chalcone 9X was equivalent to 5-FU in suppressing invasion and migration of cancer cells. Additionally, chalcone 9X has good solubility in polar solvent that is convenient in clinical applications. Therefore, chalcone 9X might be a candidate compound for the discovery of novel clinical anticancer drug.

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**Table 1**: Molecular docking results of chalcone 9X with caspase-3 and caspase-8.

**Conclusions**

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Fig. 5. Chalcone 9X inhibits invasion and migration of HepG2 cells. (A & B) Transwell assay results showing the inhibitory effects of 50 and 100 μmol/L chalcone 9X on invasion of HepG2 cells as compared to those of 30 μmol/L 5-FU. Scale bar indicates 50 μm. (C & D) Wound healing assay results demonstrating the inhibitory effects of 50 and 100 μmol/L chalcone 9X on migration of HepG2 cells as indicated by the slower closing of scratch wounds. Scale bar indicates 50 mm. The data are presented as mean ± SEM. n = 3. *p < 0.05, **p < 0.01, ***p < 0.001 vs. Control.

Fig. 6. Chalcone 9X inhibits invasion and migration of H460 cells. (A & B) Transwell assay results showing the inhibitory effects of 50 and 100 μmol/L chalcone 9X on the invasion of H460 cells. Scale bar indicates 50 μm. (C & D) Wound healing assay depicting the inhibitory effects of 50 and 100 μmol/L chalcone 9X on migration of H460 cells as indicated by the slower closing of scratch wounds. Scale bar indicates 50 mm. The data are presented as mean ± SEM. n = 3. *p < 0.05, **p < 0.01, ***p < 0.001 vs. Control.
Fig. 7. Chalcone 9X inhibits tumor growth in a mouse model of xenograft tumors. (A) Experimental protocol of administration of chalcone 9X and 5-FU. (B) The tumor growth curves comparing the differences of tumor size among the varying groups. The data are presented as mean ± SEM. n = 3-6. *p < 0.05, **p < 0.01, ***p < 0.001 vs. Control.

Fig. 8. Chalcone 9X alters expression of apoptosis-related proteins in tumor tissues from xenograft mice. (A) Lack of significant effects on protein level of total caspase-3 by chalcone 9X at three different dosages, as assessed by western blot analysis. (B) Lack of significant effects on protein level of total caspase-8 by chalcone 9X at three different dosages. (C) Downregulation of protein level of Bcl-2 by chalcone 9X at three different dosages. (D) Upregulation of protein level of cleaved caspase-3 by chalcone 9X at three different dosages as assessed by western blot analysis using 5-FU as a positive control agent. (E) Upregulation of protein level of cleaved caspase-8 by chalcone 9X at three different dosages. (F) Upregulation of protein level of Bax by chalcone 9X at three different dosages. The data are presented as mean ± SEM. n = 3. *p < 0.05, **p < 0.01, ***p < 0.001 vs. Control.
**References**


**Conflict of interest**

None of our authors has any conflict of interest to be explored.

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**Table 1**

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**Fig. 9.** In silico molecular docking of chalcone 9X and caspase-3 and -8 proteins. (A & C) The virtual binding mode of chalcone 9X in the active site of caspase-3 (PDB: 2J30). (B & D) The virtual binding mode of chalcone 9X in the active site of caspase-8 (PDB: 3kjn). Chalcone 9X is represented as ball-stick diagrams with carbon atoms shown in grey, hydrogen atoms shown in blue and oxygen atoms shown in red. (A & B) Relevant amino acid residues in the binding site are shown as stick diagrams. Yellow dashed lines represent H-bonds. (C & D) Chalcone 9X is surrounded by lipophilic surface of targets. Brown represents the most hydrophobic regions, and blue the most hydrophilic domains.