RESEARCH ARTICLE

Iso-suillin Isolated from *Suillus luteus*, Induces G_1 Phase Arrest and Apoptosis in Human Hepatoma SMMC-7721 Cells

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Abstract

Iso-suillin, a natural product isolated from *Suillus luteus*, has been shown to inhibit the growth of some cancer cell lines. However, the molecular mechanisms of action of this compound are poorly understood. The purpose of this study was to investigate how iso-suillin inhibits proliferation and induces apoptosis in a human hepatoma cell line (SMMC-7721). We demonstrated the effects of iso-suillin on cell proliferation and apoptosis in SMMC-7721 cells, with no apparent toxicity in normal human lymphocytes, using colony formation assays and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) analysis. Western blotting was used to examine the expression of G_1 phase-regulated and apoptosis-associated protein levels in iso-suillin treated SMMC-7721 cells. The results indicated that iso-suillin significantly decreased viability, induced G_1 phase arrest and triggered apoptosis in SMMC-7721cells. Taken together, these results suggest the potential of iso-suillin as a candidate for liver cancer treatment.

Keywords: Iso-suillin - G_1 phase arrest - apoptosis - SMMC-7721 cells

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Introduction

Hepatocellular carcinoma (HCC) is a primary malignancy of the liver. The incidence of HCC is highest in Asia, particularly in China and Japan. In China, 50 people per 100, 000 are affected annually. HCC is now the second leading cause of cancer death in China (Parkin et al., 2005; El-Serag et al., 2007). The efficacy of clinical drugs at present is limited by a range of adverse side-effects such as toxicity and drug resistance, although substantial advances have been made in the chemotherapy of HCC. There is still an urgent need to explore more effective chemotherapeutic agents against this disease.

In China, mushrooms have been used as traditional foods and medicines for a long time. There are various classes of primary and secondary metabolites in mushrooms which exhibit significant anti-cancer, immunomodulatory, anti-microbial and antiviral effects (Roupas et al., 2012). Therefore, they represent a valuable source of novel chemotherapeutic agents. Despite the potential for drug development, only a few bioactive metabolites from mushrooms have been reported so far as compared with those from higher plants and microbes. Prenylphenols are a class of natural products. A number of phenolic compounds have been isolated from Albatrellus ovina, including the novel neogrifolin derivatives 3-hydroxyneogrifolin, 1-formylneogrifolin and 1-formyl-3-hydroxyneogrifolin along with grifolin (Ye et al., 2005) and neogrifolin (Nukata et al., 2002; Yuen-Nei Cheung et al., 2005), scutigeral, ilicicolin B, ovinal and ovinol (Dekermendjian et al., 1997) and suillin (Liu et al., 2009). These compounds have been shown to play important physiological roles, including antioxidant activity, cholesterol metabolism regulation, antibacterial activity, anti-tumor activity and anti-inflammatory activity. Hence, prenylphenols have an excellent potential for development as drugs.

We isolated a suillin isoform from a petroleum ether extract of Suillus flavus, called iso-suillin, which also belonged to the prenylphenol class (Figure 1). Geraci et al. (Geraci et al., 1992) found that iso-suillin could efficiently inhibit the growth of KB cells, P-388 cells and NSCLC-N6 cells. However, the mechanism of anti-tumor action was not further studied.

In this study, we investigated the effects of iso-suillin on cell proliferation and apoptosis and examined the expression of the proteins associated with cell cycle regulation and apoptosis in SMMC-7721 cells.

Materials and Methods

Materials

Human hepatoma cell line SMMC-7721 was acquired from Hebei Medical University Cell Biology Department for cancer research. Iso-suillin was prepared in the Fungus Laboratory of the College of Life Sciences, HeBei Normal University. The cells were cultured in RPMI-1640 medium (Gibco, USA), supplemented with 10% heat-inactivated fetal bovine serum (Gibco, USA) and 1% (v/v) penicillin-streptomycin (Gibco, USA) in a 37°C, 5% CO_2 humidified

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incubator (HF90, Heal Force, China). Antibodies against caspase-3, -8, -9, cytochrome C, FADD were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Cyclin D1, Cyclin E1, CDK2, CDK4, E2F-1, p21, p53, p-Rb, β-actin and secondary antibody conjugated to horseradish peroxidase were obtained from Bioworld Technology (Bioworld Technology, Atlanta, GA, USA).

Purification and identification of iso-suillin

Fresh Suillus flavus fruiting bodies were collected from the Taihang Mountains of China. The dry material of Suillus flavus was extracted with petroleum ether and was stirred for 2 h at room temperature. After filtration, the solution was evaporated in vacuo to a residue. This residue was subjected to column chromatography (CC) (SiO2, petroleum ether/AcOEt 50:1; 40:1; 30:1; 20:1; 10:1; 8:1; 5:1; and 1:1), producing nine fractions. Fraction 7 (eluted with petroleum ether/AcOEt 8:1) was repurified by CC (SiO_2, petroleum ether/AcOEt 3:1; and Sephadex LH-20, $CHCl_3$/MeOH 1:1) and was confirmed by high performance liquid chromatography (HPLC) to possess a purity of 99.0%. The compound isolated from fraction 7 had a molecular formula of C28H40O4 and a relative molecular weight of 440.29. Its structure was identified by a combination of spectroscopic analysis. As an isomer of suillin, we named it iso-suillin.

Cell viability assay

The exponential growth phase of SMMC-7721 was planted into 96-well multiplates and after adhesion the cells were treated with series concentrations of iso-suillin. Cells were added to MTT reagent in phosphate buffered saline (PBS) at a final concentration of 0.5 mg/mL, and then incubated for 4 h at 37°C. 150 μL DMSO was added by shaking for 10 min in the dark. The result was quantified by measuring the absorbance at 490 nm though a multilabel counter (BioTek, USA).

Colony formation assay

SMMC-7721 cells were treated with series concentrations of iso-suillin for 48 h. After treatment, the cells were suspended and re-seeded into 6-well plates at a density of 200 cells per well. After two weeks, cells were fixed using 4% paraformaldehyde (PFA) and stained with Crystal Violet Staining Solution. The visible colonies (≥50 cells) were counted and typical images were photographed using a common Nikon camera.

Observation of morphologic changes

SMMC-7721 cells were seeded into 6-well plates and exposed to the indicated concentration of iso-suillin for 48 h. The cells were stained with 4', 6-diamidino-2-phenylindole (DAPI [Sigma, USA]). Cellular morphology was observed using a fluorescence microscope (Nikon, Tokyo, Japan). Apoptosis detection was completed in the Hebei Medical University Electron Microscope Room using transmission electron microscopy (H-7500, Hitachi).

Apoptosis analysis by Annexin-FITC/PI

After 48 h iso-suillin treatment, the cells were harvested and washed twice with ice-cold phosphate buffered saline (PBS). The level of apoptosis was determined using an Annexin V-FITC /PI apoptosis detection kit (Invitrogen, USA) according to the manufacturer's instructions. 2.7. Flow cytometry analysis of cell cycle Cells were treated with 0, 1.4, 2.8 and 5.6 μM final concentrations of iso-suillin for 48 h, and then the cells were harvested, fixed in 75% ethanol at 4°C overnight and washed twice with ice-cold PBS, followed by incubation with RNase and propidium iodide, a DNA-intercalating dye. Cell cycle analysis was done with flow cytometry (FCM [Epics- XL II, Beckman Coulter, USA]).

Analysis of the mitochondrial membrane potential

The change in mitochondrial membrane potential (MMP) after iso-suillin treatment was analyzed by FCM using rhodamine (Rh)-123 (Sigma, USA) staining. Then the cells were stained in PBS containing 3 μg/mL Rh-123 at 37°C in the dark for 30 min. The stained cells were then washed with ice-cold PBS, and the Rh-123 fluorescence was detected by FCM (Epics- XL II, Beckman Coulter, USA).

Protein extraction and Western blotting

For the analysis of protein expression, SMMC-7721 cells were treated with serum-free 1640 medium (blank) or with iso-suillin at 0, 1.4, 2.8 and 5.6 μM and processed 48 h after incubation. Then the cells were washed twice with ice-cold PBS and incubated with lysis buffer for 30 min at 4°C. Protein concentration in cell lysates was measured by the Bradford method. Sixty micrograms of total proteins were subjected to 6-12% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto nitrocellulose membranes (Millipore, Burlington, MA, USA). After blocking with 5% nonfat milk, the membranes were washed with PBS containing 0.1% Tween 20 and incubated with primary antibodies followed by respective horseradish peroxidase-conjugated secondary antibodies. Signals were detected with electrochemiluminescence (ECL [Pierce, USA]) reagents. β-actin was used as a loading control.

Statistical analyses

Data were presented as mean ± SD, and results were analyzed using SPSS16.0 software (IBM, USA). The significance of difference was determined using a one-way ANOVA test. Values of $P < 0.05$ were considered to be statistically significant.

Results

Iso-suillin decreases cell viability in the human hepatoma cancer cell line SMMC-7721

Geraci et al. (Geraci, Piattelli, Tringali, Verbist, & Roussakis, 1992) found that iso-suillin could efficiently inhibit the growth of KB cells, P-388 cells and NSCLC-N6 cells. In this study, the human hepatoma cancer cell line SMMC-7721 was used to detect the anti-proliferative potential of iso-suillin. The results are shown in Figure 1. The cells were treated with different concentrations of iso-suillin for 24, 48, or 72 h, and MTT assay showed iso-suillin significantly suppressed SMMC-7721 cell

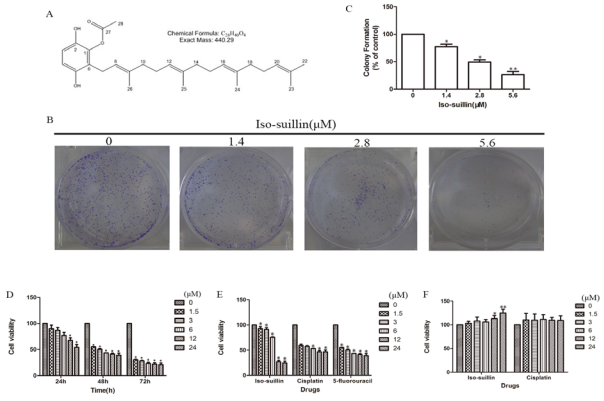

Figure 1. Proliferation Inhibition Caused by Iso-suillin Treatment in SMMC-7721 Cells. (A) The chemical structural formula of iso-suillin. (B) Representative photographs of SMMC-7721 cells treated with various concentrations of iso-suillin for 48 h and stained with 4%PFA and crystal violet. (C) Graphical analysis of the results presented in (B). (D) Cell viability after treatment with iso-suillin for 24, 48 or 72 h. (E) Cell viability after treatment with iso-suillin, cisplatin or 5-fluorouracil for 48 h. (F) The effect of iso-suillin and cisplatin on cell viability in normal human lymphocytes. Cell viability was determined by MTT assay. Data are expressed as mean ± SD, n = 3. **P*<0.05, ***P*<0.01, compared with the control

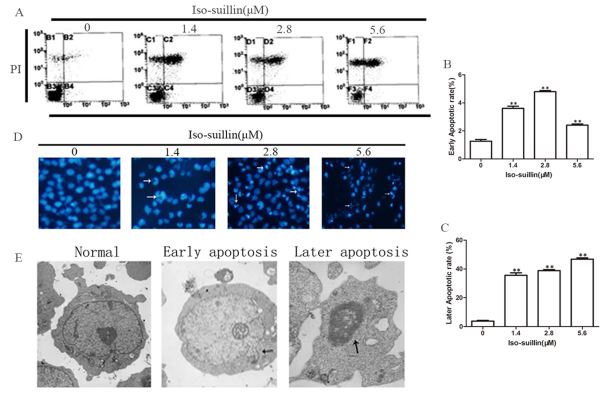

Figure 2. Iso-suillin-induced Apoptosis in SMMC-7721 Cells. (A) SMMC-7721 cells treated with different concentrations of iso-suillin for 48 then stained with Annexin-FITC/PI and analyzed by flow cytometry. (B-C) Rates of early and late apoptosis in SMMC-7721 cells, induced by increasing concentrations of iso-suillin. (D) Iso-suillin-induced apoptosis in SMMC-7721 cells as examined using DAPI staining. Photographs were taken using fluorescence microscopy (200×). Scale bar, 50 μm. (E) Observation of morphologic changes using electron microscopy. Scale bar, 500 nm. Data expressed as mean ± SD of three independent assays. **P*<0.05, ***P*<0.01

proliferation and viability in a concentration-dependent manner, with time-dependent inhibition. IC50 values were 26.1, 2.8 and 1.2 μM for 24, 48 and 72 h respectively (Figure 1D). Cisplatin and 5- fluorouracil are common chemotherapy drugs, so we also investigated cell viability after iso-suillin, cisplatin and 5- fluorouracil incubation for 48 h. The results showed that all three drugs could significantly inhibit SMMC-7721 cell viability (Figure 1E). For normal human lymphocytes, there was no obvious inhibitory effect observed after iso-suillin or cisplatin incubation for 24 h (Figure 1F). To further investigate the anti-cancer effect of iso-suillin in human SMMC-7721 cells, the colony formation assay was used. The number

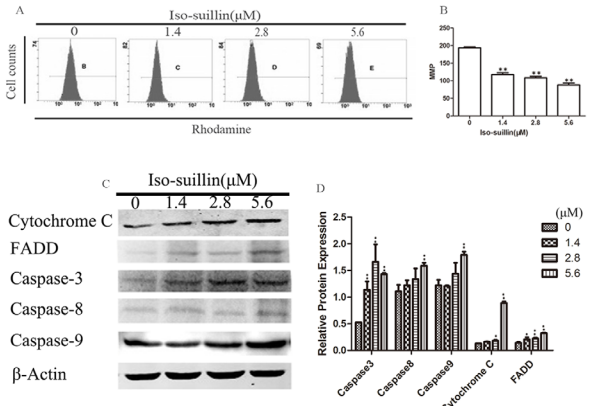

Figure 3. Induction of Apoptosis in SMMC-7721 Cells by Iso-suillin. (A) The effect of iso-suillin on mitochondrial membrane potential (MMP). (B) MMP of iso-suillin treated cells calculated using the formula lg (X-Mode) ×340. (C) Western blot analysis using lysates from SMMC-7721cells treated with iso-suillin for 48 h. β-actin was used as an internal control. (D) Columns show relative protein expression compared with equal loading control. Data are expressed as mean ± SD, n = 3. **P*<0.05, ***P*<0.01, compared with control, one-way analysis of variance

of colony foci showed a dose-dependent decrease after treatment with 0, 1.4, 2.8 and 5.6 μM iso-suillin (Figure 1B, C).

Iso-suillin induced morphological changes and apoptosis in SMMC-7721 cells

An additional investigation was carried out to determine the cell morphology, and to quantify different stage apoptotic cells. SMMC-7721 cells were treated with 0, 1.4, 2.8 and 5.6 μM iso-suillin for 48 h. We measured phosphatidylserine exposure using Annexin V-FITC/PI staining by FCM. Figure 2A indicates that the proportion of annexin V stained cells increased significantly. Figures 2B and 2C show the rates of early apoptosis and late apoptosis of SMMC-7721 cells. The results indicated that early apoptosis and late apoptosis significantly increased with increased iso-suillin concentration. We also conducted DAPI staining and electron microscopy to observe morphologic changes. When SMMC-7721 cells were treated with different concentrations of iso-suillin for 48 h, followed by DAPI staining, as shown in Figure 2 D, iso-suillin induced cell morphological changes and decreased cell numbers. Cells also became smaller, round and blunt and showed nuclear fragmentation when compared with control SMMC-7721 cells. After treatment of cells with iso-suillin for 48 h, we observed the cells using transmission electron microscopy. We found that in normal cells, the plasma membrane, nuclear envelope and nucleolus were complete and distinct, while the treated cells exhibited shrinkage, chromatic agglutination, nuclear and plasma membrane convolution (Figure 2 E). The results indicated that the cytotoxic action of iso-suillin was due to its ability to induce apoptosis.

Iso-suillin decreased the mitochondrial membrane potential and affected the apoptosis-associated protein levels in SMMC-7721 cells

To investigate the mechanisms of iso-suillin induced

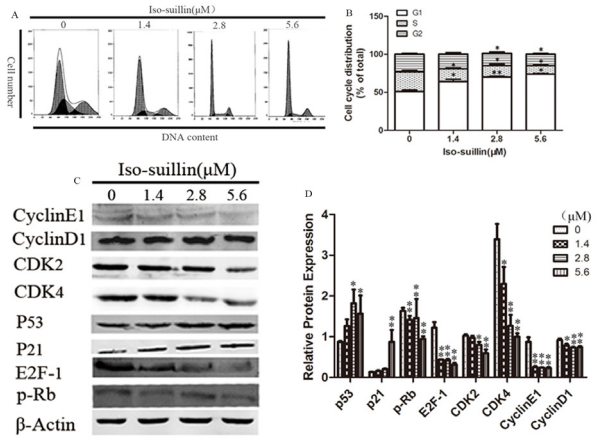

Figure 4. Iso-suillin Induces G1 Cell Cycle Arrest and Affects the Levels of G1 Regulatory Proteins. (A) Logarithmically growing cells were treated with 1.4, 2.8, and 5.6 μM iso-suillin for 48h. Cells were fixed with 70% ethanol, stained with propidium iodide, and subjected to flow cytometry analysis to determine DNA content. (B) Columns show the percentage of SMMC-7721 cells in each phase of the cell cycle after treatment with iso-suillin for 48 h. (C) Regulation of the expression of cyclinD1, cyclinE1, CDK2, CDK4, p53, p21, E2F-1, and p-Rb by iso-suillin. SMMC-7721 cells were treated with iso-suillin for 48 h and the cell lysates were analyzed using Western blotting. (D) Columns show relative protein expression compared with equal loading control. Data are expressed as mean ± SD, n = 3. **P*<0.05, ***P*<0.01, compared with control, one-way analysis of variance

apoptosis in SMMC-7721cells, we examined the mitochondrial membrane potential using rhodamine 123 staining. Cells were treated with 0, 1.4, 2.8 and 5.6 μM iso-suillin for 48 h, then the cells were stained with rhodamine 123 and analyzed by FCM. As shown in Figures 3A, and 3B, the mitochondrial membrane potential decreased remarkably when treated with 1.4 μM iso-suillin and this became more pronounced when treated with 2.8 and 5.6 μM iso-suillin. Changes in apoptosis-associated protein levels were determined by western blotting. Results in Figures 3C and 3D indicate that iso-suillin increased the levels of cytosolic cytochrome C, and caspase-3, -8 and -9.

Iso-suillin induced cell cycle arrest and affected associated protein levels in SMMC-7721 cells

Based on the results from growth inhibition, further studies were conducted to investigate the possible mechanisms involved in iso-suillin-induced cell cycle arrest and associated protein levels in SMMC-7721 cells in vitro. The results from flow cytometric assay revealed that 48 h iso-suillin treatment induced accumulation of the G_0/G_1 phase in SMMC-7721 cells and this effect was dose-dependent (Figures 4A, 4B). Western blotting also showed that iso-suillin down-regulated the expression of cyclin D1 and cyclin E1, CDK2 and CDK4, p-Rb and E2F-1 and up-regulated the expression of p21, p53 (Figures 4C, 4D).

Discussion

Many studies have shown an association of abnormal cell cycle regulation and apoptosis with cancer, and cell cycle inhibitor and apoptosis-inducing agents are being appreciated as weapons for the management of cancer (Evan et al., 2001; Schmitt et al., 2003). In the present study, we first demonstrated that iso-suillin not only significantly inhibited the viability of SMMC-7721 cells as well as cisplatin and 5-fluorouracil, and obviously decreased the number of colony foci (Figure 1), but also induced morphological changes and apoptosis (Figure 1). We also found that iso-suillin did not affect lymphocyte proliferation at low concentrations but promoted lymphocyte proliferation at higher concentrations (Figure 1). The effects of iso-suillin were specific for tumor cells, and no cytotoxicity toward normal cells was observed. These results suggested that iso-suillin had the potential to be a novel treatment for liver cancer.

Anticancer agents may alter regulation of the cell cycle machinery, resulting in an arrest of cells in different phases of the cell cycle, thereby reducing growth and proliferation (An et al., 2013). Since iso-suillin induced a remarkable viability decrease in SMMC-7721 cells, we investigated whether this effect was due to cell cycle arrest. Analysis of the cell cycle by flow cytometry indicated that treatment of cells for 48 h with iso-suillin increased the percentage of cells in the G_0/G_1 phase and decreased the percentage of cells in G_2 and S phases (Figure 4). This suggests that iso-suillin inhibits cell proliferation by inducing cell cycle arrest in the G_0/G_1 phase.

The function of cell cycle regulatory proteins, such as cyclins and CDKs (cyclin-dependent kinases), is an important means of inhibition of cancer cell growth and division (Chen et al., 2002). Transit through G_1 into S phase requires the activation of cyclinD and cyclinE. Cyclin D1 is expressed at high levels in the middle and at the end of the G_1 phase of the cell cycle. High levels of cyclin D1 in G_1 promote cell entry into S phase. Down-regulation of this marker indicates cell cycle progression arrest and in some cases may result in cell death through apoptosis (Baker et al., 2005). Meanwhile, p-Rb is an important regulator of genes responsible for progression through the G_1 phase, and it can disrupt complexes with E2Fs, allowing cell cycle progression into the S phase (Hume et al., 2008). Cyclin E, CDK2 and CDK4 are also important complexes responsible for the progression of cells through the G_1 phase of cell cycle and initiation of DNA replication (Sherr et al., 2004). These proteins are all important for G_1 cell cycle progression (Moeller et al., 2006). In the present study, we found iso-suillin was able to decrease the expression of cyclinD1, cyclinE1, CDK2, CDK4, p-Rb and E2F-1 (Figure 4C and 4D). These results indicated some mechanisms by which iso-suillin can inhibit the expression of these regulatory proteins in the course from G_1 to S transition.

P53 is a key element in the induction of cell cycle arrest and apoptosis following DNA damage or cellular stress in human cells (Vousden et al., 2007). Furthermore, p53 functions to prevent initiation of DNA replication in the G_1/S checkpoint and maintain G_0/G_1 arrest (Vousden et al., 2009). DNA damage is one of the molecular events associated with cell cycle arrest and apoptosis and many anti-cancer reagents induce DNA damage (Cai et al.,

2007). Recent studies demonstrate that the activation of the ATR-Chk1 pathway in response to DNA damage is strongly cell cycle-regulated (Xu et al., 2010) and the up-regulation of p-Chk1 can activate the expression of p53 (Kastan & Bartek, 2004). Cell cycle arrest which is dependent on p53 requires trans-activation of p21 or other cell cycle-related factors (Kosakowska-Cholody et al., 2005). The induction of p21 causes subsequent arrest in the G_0/G_1 or G_2/M phase of the cell cycle by binding of the cyclin-CDK complex (Gartel et al., 2005; Child et al., 2006). Obstruction of cell cycle progression in cancer cells is considered as one of the most effective strategies for the control of tumor growth (Nam et al., 2007). In the present study, we found iso-suillin could increase the expression of p53 and p21 (Figure 4C-D). These results suggest that iso-suillin might cause DNA damage in SMMC-7721 cells, and also activate p-Chk1. p-Chk1 is able to increase expression of p53, which is the activator of p21. In regulating the entry of cells to the G/S transition checkpoint, p21 plays a key role. Iso-suillin might, through p53/p21, mediate G_0/G_1 cell cycle arrest in SMMC-7721 cells.

It is well known that apoptosis is initiated via two main pathways that lead to the activation of caspases, namely the death receptor pathway and the mitochondrial pathway (Spencer et al., 2011). This study demonstrated that p53 can lead to mitochondrial-mediated and caspase-dependent apoptosis (Zhang et al., 2013). Meanwhile, p53 regulates the balance between pro-apoptotic and anti-apoptotic genes, and its activation is required for apoptosis (Vousden et al., 2009). However, our study did not explore the underlying mechanisms. Cytochrome C is released from mitochondria into the cytoplasm, which activates caspase-9/-3 and leads to cell apoptosis (Zhang et al., 2012). In addition, Fas-Associated protein with Death Domain (FADD) binds to death receptors, which in turn activates caspase-8/-3 and could also induce apoptosis (Lei et al., 2012). Caspases play a pivotal role in the execution of programmed cell death (Sun et al., 2013). Caspase-8 and caspase-9 are the initiator caspases and caspase-3 is the "executioner enzyme" (Wang et al., 2005). The activated caspase-3 leads to final destruction of the target cell (Zhang et al., 2012). In our investigation, iso-suillin decreased mitochondrial membrane potential significantly, and simultaneously increased the expression of caspase-3, -8, -9, cytochrome C and FADD in SMMC-7721cells (Figure 3C and 3D). These results suggest that the death-receptor signaling pathway and mitochondrial pathway may mediate the iso-suillin induced apoptotic response in SMMC-7721 cells.

This study is the first to report the effects of iso-suillin on liver cancer cell cycle arrest and apoptosis. The results demonstrated that iso-suillin could selectively arrest SMMC-7721 cell growth at the G_0/G_1 checkpoint. We conclude that iso-suillin induces apoptosis in SMMC-7721 cells via the mitochondrial and death-receptor pathways and that iso-suillin mediated cell cycle arrest is related to the expression of p53. These data provided mechanistic insights into iso-suillin inhibition effects on SMMC-7721 cells, which suggest iso-suillin may be a promising anticancer agent.

Acknowledgements

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