Iso-suillin from *Suillus flavus* Induces Apoptosis in Human Small Cell Lung Cancer H446 Cell Line

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Abstract

Background: The suillin isoform iso-suillin is a natural substance isolated from a petroleum ether extract of the fruiting bodies of the mushroom *Suillus flavus*. Previous studies have found its inhibition effect on some cancer cells, and we aimed to study its effects on human small cell lung cancer H446 cell line.

Methods: Cell viability was measured by 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2-H-tetrazolium bromide assay. Cellular morphological changes (apoptosis and necrosis) were evaluated using an electron microscope and Hoechst 33258 staining detected by the inverted microscope. Flow cytometry was used to detect cell apoptosis, cell cycle distribution, and mitochondrial membrane potential. Protein expression was determined by Western blotting analysis.

Results: Here, we describe the ability of iso-suillin to inhibit the growth of H446 cells in time- and dose-dependent way. Iso-suillin had no obvious impact on normal human lymphocyte proliferation at low concentrations (9.09, 18.17, or 36.35 μmol/L) but promoted lymphocyte proliferation at a high concentration (72.70 μmol/L). After treatment of different concentrations of iso-suillin (6.82, 13.63, or 20.45 μmol/L), the apoptosis rate of H446 cells increased with increasing concentrations of iso-suillin (16.70%, 35.54%, and 49.20%, respectively, all \(P < 0.05\) compared with the control), and the expression of related apoptotic proteins in the mitochondrial pathway including cytochrome c and caspase-9 were up-regulated compared with the control (all \(P < 0.05\)). On the contrary, Bcl-2/Bax ratio was down-regulated compared with the control. Besides, the expression of pro-apoptotic proteins in the death receptor apoptosis pathway, including Fas-associated protein with a novel death domain and caspase-8, and the expression of caspase-3, a downstream regulatory protein of apoptosis, were also increased compared with the control (all \(P < 0.05\)). Inhibitors of caspase-9 and caspase-8 reversed the apoptosis process in H446 cells to varying degrees.

Conclusions: These results suggest that iso-suillin could induce H446 cell apoptosis through the mitochondrial pathway and the death-receptor pathway. Therefore, iso-suillin might have a potential application as a novel drug for lung cancer treatment.

Key words: Apoptosis; Death Receptor Pathway; H446 Cells; Iso-suillin; Mitochondrial Pathway

Introduction

In recent years, research and development of metabolites from plants and macro-fungi, with novel structures and biological activities, have gained extensive attention for their potential development as drugs.³⁴ Prenylphenols have been of particular interest: they exist not only in plants (e.g., vanilloids),³¹ but also in macro-fungi, with examples including grifolin,³² neo-grifolin,³³ and suillin.³⁴ Prenylphenols have been reported to play important physiological roles in antioxidant activity,³⁴ cholesterol metabolism regulation, antibacterial activity,³⁵ antitumor activity,³⁶ anti-inflammatory activity,³⁷ dopamine D1 receptor binding, and vanilloid receptor modulation activity.³⁸ Hence, prenylphenols have excellent potential to be further developed as drugs. Among the prenylphenols, grifolin and suillin have been further investigated.

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Tringali et al.\cite{9} isolated suillin from dichloromethane extracts of *Suillus granulatus* and found that suillin had strong inhibitory effects on human nasopharyngeal carcinoma KB cells, human bronchial cancer nonsmall cell lung cancers (NSCLC)-N6 cells, and particularly, mouse leukemia P-388 cells. Liu et al.\cite{5} also isolated suillin from ethyl acetate extracts of *Suillus placidus* and determined its antitumor spectrum using eight cancer cell lines (HepG2, Hep3B, Huh7, Bcap37, MCF-7, HeLa, H446, and SW620). Their results indicated that suillin was most effective in inducing apoptosis of human hepatoma cells (HepG2, Hep3B, and Huh7). Further experiments indicated that suillin could induce HepG2 cell apoptosis via the mitochondrial pathway and the death receptor pathway.

Tringali et al.\cite{10} improved the isolation process and obtained several structurally analogous phenols including iso-suillin, an isomer of suillin [Figure 1a]. They found that iso-suillin could efficiently inhibit the growth of *Escherichia coli* and *Micrococcus*. Later, Geraci et al.\cite{11} studied the relationship between iso-suillin structure and cytotoxicity, and found that iso-suillin could efficiently inhibit the growth of KB cells, P-388 cells, and NSCLC-N6 cells.

In previous studies, iso-suillin was isolated from petroleum ether extracts of *Suillus flavus*. We found that iso-suillin could not only efficiently inhibit cell proliferation, but also induce apoptosis in K562 cells and SMMC-7721 cells.\cite{12,13} Lung cancer is one of the major health issues all over the world, especially in China, and the situation is getting serious.\cite{14} In the present study, we examined the effects of iso-suillin on cell proliferation and apoptosis in human small cell lung cancer H446 cells.

**Methods**

**Materials and cell culture**

Iso-suillin was prepared in Fungus Laboratory of the College of Life Sciences, Hebei Normal University. Its purity was verified to be >97% by high-performance liquid
chromatography. BGC-823, SMMC-7721, Hela, MCF-7, and H446 cell lines were preserved in liquid nitrogen in College of Basic Medical, Hebei Medical University and were cultured using RPMI 1640 medium, supplemented with 10% fetal bovine serum, 2 mmol/L L-glutamine, 100 U/ml of penicillin, and 100 μg/ml of streptomycin in a 37°C humidified incubator with 5% CO₂.

**Cell viability assay**

Cell viability was determined by 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2-H-tetrazolium bromide (MTT) assay as previously described. Exponentially growing H446 cells were planted into 96-well plates at a density of 1 × 10⁵/ml. After 24 h incubation, cells were treated with serial concentrations of iso-suillin. The cells were cultured for different time periods (24 h, 48 h, and 72 h). The plate samples were read at 570 nm on a microplate reader (Elx800, BioTek, USA). The inhibition rate and the IC₅₀ value of iso-suillin on cells were calculated.

Lymph cells from 5 ml venous blood were prepared with separating medium. Lymph cells were planted into 96-well plates (2 × 10⁶/ml). After culturing for 24 h, iso-suillin was added at concentrations of 0, 9.09, 18.17, 36.35, and 72.70 μmol/L, respectively. Cell culture and the MTT test were then carried out as for the previous experiment.

**Morphological examination**

H446 cells were cultured as described above, and then 1 ml samples of cell suspension were planted into 24-well plates at a density of 1 × 10⁵/ml. After 24 h, the cells were treated with iso-suillin at concentrations of 0, 6.82, 13.63, or 20.45 μmol/L. The volume of culture medium was kept at 2 ml. Each group had three wells. The cells were cultured for 48 h before Hoechst 33258 (10 μg/ml, Sigma, USA) staining and observed under an inverted microscope (IX51, Olympus, Tokyo, Japan).

H446 cells were prepared for electron microscopy by collecting them in the logarithmic phase and dispensing them into groups. The experimental group contained 5 ml iso-suillin with a final concentration of 13.63 μmol/L and the control group had the same amount of serum-free medium. After culturing for 48 h, the cells were collected and fixed with 4% glutaraldehyde. Transmission electron microscopy (H-7500, Hitachi, Ltd., Tokyo, Japan) was carried out in the Hebei Medical University Electron Microscope Room.

**Flow cytometry assay**

After treatment with iso-suillin for 48 h, the cells were double stained with an Annexin V-FITC/PI Kit (MultiSciences Biotech, China) according to the manufacturer’s instructions. The apoptosis rate and cell cycle distribution were analyzed using flow cytometry (Epics-XL II, Beckman Coulter, USA).

**Measurement of mitochondrial membrane potential**

Cells were gently mixed with 500 μl rhodamine123 (10 μmol/L) (Sigma, USA) and stained at 37°C in a water-bath in the dark for 20 min. After washing with D-Hanks buffer, the cells were centrifuged, and the supernatants were discarded. The cell pellets were re-suspended in 1 ml D-Hanks buffer solution, and the mitochondrial membrane potential (MMP) was measured with flow cytometry.

**Western blotting analysis**

Cells were treated with iso-suillin at concentrations of 6.82, 13.63, and 20.45 μmol/L for 48 h. Then, cells were collected for protein extraction. Total protein was lysed using radio immunoprecipitation assay lysis buffer. The protein used for cytochrome c detection was extracted from cytosol using a Nuclear and Cytoplasmic Protein Extraction Kit (Beyotime, Shanghai, China).

Antibodies (Cell Signaling Technology, USA) for β-actin, cytochrome c, cleaved caspase-9, Fas-associated protein with death domain (FADD), cleaved caspase-8, caspase-3, Bcl-2, and Bax were incubated overnight at 4°C. The membrane was then incubated with goat anti-rabbit secondary antibody (Bioss, China) at room temperature for 60 min. Enhanced chemiluminescence kit (CWbio, China) was used before analyzing with a chemiluminescence imaging system (Image Reader Las-4000, Fuji, Japan).

**Effects of apoptotic pathway inhibitors**

Cells were cultured, treated, and processed as described above. After culturing for 24 h, the collected cells were divided into different groups. For each experimental treatment, a total volume of 6 ml solution, which contained different concentrations and combinations of iso-suillin, Z-IE TD-FMK (a caspase-8 inhibitor) (R&D, USA), and Z-LEHD-FMK (a caspase-9 inhibitor) (R&D) were added into the culture medium. The cells were cultured for another 48 h before double staining with the Annexin V-FITC/PI kit according to the manufacturer’s instructions. The apoptosis rate was determined using flow cytometry (Epics-XL II, Beckman Coulter, USA).

**Data analysis**

Data were analyzed using one-way analysis of variance (ANOVA) and Dunnett t-test with STATISTICA 6.0 software (Statsoft Inc., Tulsa, OK, USA), and statistical significance was determined as P < 0.05. All experiments were repeated at least three times, and the data were presented as a mean ± standard deviation (SD).

**Results**

**Anti-cancer spectrum and inhibition of H446 cells**

The inhibition of cell proliferation by different levels of iso-suillin was determined in five kinds of cancer cell, and the IC₅₀ values were calculated. The IC₅₀ value from low to high was in the following order: H446 (9.54 μmol/L), BGC-823 (11.60 μmol/L), SMMC-7721 (42.04 μmol/L), Hela (47.79 μmol/L), and MCF-7 (77.31 μmol/L). The comparison between IC₅₀ of iso-suillin and cisplatin in different cell lines on 48 h is shown in Table 1. This result indicated that H446 cells were the most sensitive to iso-suillin. Compared with the IC₅₀ of cisplatin (14.82 μmol/L), the effect of iso-suillin (9.54 μmol/L) was superior to cisplatin...
on H446 cells. The inhibition rates of iso-suillin on H446 cell proliferation are shown in Figure 1b. With increasing iso-suillin exposure time and concentration, the inhibition rate significantly increased in a time- and dose-dependent manner.

MTT results showed that iso-suillin had a little impact on normal human lymphocyte proliferation at low concentrations (<36.35 μmol/L) but could promote lymphocyte proliferation at high concentrations (>36.35 μmol/L). These results suggest that the effect of iso-suillin on cancer cells could be specific, with no anti-proliferative effect on normal lymphocyte [Figure 1c].

To investigate the effects of iso-suillin on cell cycle distribution, H446 cells were treated with different concentrations of iso-suillin for 48 h [Figure 1d]. The flow cytometry results showed, in the untreated control cells, the highest percentage of cells were in the S phase, followed by the G2/M phase, with apoptosis being the late event in the apoptotic process. In this study, MMP was measured with flow cytometry analysis.

![Figure 2](image)

**Figure 2: Microscopic observation of the apoptotic characteristics of iso-suillin-treated H446 cells.** H446 cells were treated with iso-suillin at different concentrations (0, 6.82, 13.63, and 20.45 μmol/L) and observed under an inverted fluorescence microscope (original magnification, ×400) after Hoechst 33258 staining. The H446 cells treated with 0 μmol/L and 13.63 μmol/L were also observed using electron microscopy (original magnification, ×20,000). (a) Reveals the blank control cells have large, round, evenly stained nuclei. (b-d) H446 cells treated with increasing iso-suillin concentrations, 6.82, 13.63, and 20.45 μmol/L for 48 h, respectively, with nuclei displaying chromatin condensation and apoptotic bodies. (e) Reveals the normal nucleus of an H446 cell under the electron microscope. (f and g) Cells treated with 13.63 μmol/L iso-suillin, exhibiting chromatin condensation and marginalization, with apoptosis bodies deeply stained.

**Induction of apoptosis**

The apoptosis rates of H446 cells treated with iso-suillin are shown in Figure 3. After culturing for 48 h, the rates of early and late apoptosis of cells treated with different concentrations of iso-suillin gradually increased with increasing iso-suillin concentrations. Starting from 20.45 μmol/L iso-suillin, though the early apoptosis rate began to decrease, the late apoptosis rate showed an obvious increase compared with the control (all P < 0.05).

Morphological assay of cell death was also investigated using Hoechst 33258 staining. H446 cells cultured for 48 h without iso-suillin were actively proliferating, showing large, round nuclei stained evenly as shown in Figure 2a. Figure 2b-2d show that with increasing iso-suillin concentrations (6.82, 13.63, and 20.45 μmol/L), the chromatin displayed chunk, crescent, or ring shapes, indicating that the cells were undergoing apoptosis. Changes in the nuclei of H446 cells treated with iso-suillin are investigated using transmission electron microscopy as shown in Figure 2e-2g. The untreated H446 cells had a sharp-edged nucleus and obvious nucleolus. In treated H446 cells, the chromatin became condensed and marginalized, the nuclear envelope disappeared, and apoptotic bodies appeared. The results suggest that iso-suillin could induce marked apoptotic morphology in H446 cells.

**Loss of mitochondrial membrane potential**

In some apoptotic systems, loss of MMP may be an early event in the apoptotic process and an inducer for the release of apoptosis-inducing factor. In this study, MMP was measured with flow cytometry analysis. Changes in MMP of H446 cells treated with iso-suillin at different concentrations for 48 h are shown in Figure 4. As the iso-suillin concentration increased, the MMP of

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Table 1: The IC<sub>50</sub> of iso-suillin and cisplatin for different cell lines after 48 h

| Chemicals | H446  | BGC-823 | SMMC-7721 | Hela  | MCF-7  |
|-----------|-------|---------|-----------|-------|--------|
| Iso-suillin | 9.54 ± 0.65* | 11.60 ± 0.98 | 42.04 ± 2.54* | 47.75 ± 3.22 | 77.31 ± 3.69* |
| Cisplatin  | 14.82 ± 0.97 | 11.14 ± 0.84 | 13.75 ± 1.13 | 50.19 ± 3.22 | 47.75 ± 3.28 |

*P<0.05 versus cisplatin treated group.
Activation of caspase-9, -8, and -3

The caspase family is at the heart of the apoptotic machinery and plays a key role in the execution of apoptosis. Morphological assay of apoptosis led us to hypothesize that iso-suillin might activate caspase. Hence, the activation of caspase by iso-suillin was further evaluated in H446 cells. We measured the catalytic activity of caspase-8, caspase-9, and caspase-3 after 48 h of iso-suillin treatment. The results showed that the expression levels of cleaved caspase-9, -8, and -3 increased (Figure 5a-5c) with increasing iso-suillin concentrations (6.82, 13.63, and 20.45 μmol/L). Pro-caspase-3 expression was decreased, while caspase-3 expression increased as compared with the control (all \( P < 0.05 \)), indicating that iso-suillin might induce the cleavage of pro-caspase-3 to caspase-3 and regulate the caspase signaling cascade.

To further demonstrate the involvement of caspase activation in the apoptotic effect, we investigated whether the caspase-8 inhibitor Z-IETD-FMK and caspase-9 inhibitor Z-LEHD-FMK prevented apoptosis. Compared to the control group, H446 cells treated with caspase-8 inhibitor only (10 μmol/L), caspase-9 inhibitor only (10 μmol/L), or both were largely living cells, and only a small proportion was apoptotic or necrotic cells. These results indicated that apoptosis pathway inhibitors failed to affect H446 cell proliferation. In contrast, cells treated with iso-suillin only (20.45 μmol/L) showed an apoptosis rate of 35.57%. After the combined addition of caspase-8 and caspase-9 inhibitors in the iso-suillin-treated cells, the cell apoptosis rate reduced from 35.57% to 10.15% showed a decrease of 71% as compared with the control (Figure 6) (\( P < 0.05 \)). These results indicate that these two inhibitors reversed the iso-suillin-induced apoptosis process to varying degrees.
Release of cytochrome c
Cytochrome c is localized in the mitochondrial intermembrane space and loosely attached to the surface of the inner membrane. In response to a variety of apoptosis-inducing agents, cytochrome c is released from mitochondria to the cytosol.[22,23] To examine this step in the apoptotic cell death pathway initiated by iso-suillin, we measured cytochrome c with Western blotting analysis. The result showed that with increasing iso-suillin concentrations (6.82, 13.63, and 20.45 μmol/L), the expression levels of cytochrome c increased as compared with the control (all $P < 0.05$) [Figure 7a].

Up-regulation of Fas-associated protein with death domain
FADD is an adaptor molecule that bridges the Fas-receptor, and other death receptors to caspase-8 through its death domain to form the death-inducing signaling complex during apoptosis.[24,25] The data showed that expression levels of FADD were up-regulated compared with the control (all $P < 0.05$) [Figure 7b].

Up-regulation of bax and down-regulation of Bcl-2
Bcl-2 family proteins, including anti-apoptotic members (such as Bcl-2) and pro-apoptotic members (such as Bax), play a pivotal role in apoptosis.[26,27] After iso-suillin treatment, the data showed that with increased iso-suillin concentration, Bax expression increased while Bcl-2 expression decreased, and the ratio of Bax/Bcl-2 increased accordingly [Figure 7c] compared with the control (all $P < 0.05$). The results indicated that iso-suillin could induce H446 cells into apoptosis by up-regulating Bax expression and down-regulating Bcl-2 expression.

Discussion
Drugs that inhibit the proliferation and induce apoptosis in cancer cells usually show good prospects for clinical...
Hela cells, and MCF-7 cells. Iso-suillin’s inhibition effects in H446 cells (IC_{50}: 9.54 μmol/L) and BGC-823 cells (IC_{50}: 11.60 μmol/L) are close to that of cisplatin (IC_{50}: 14.82 μmol/L). Moreover, iso-suillin inhibited H446 cells growth in a dose- and time-dependent manner. The flow cytometry results also showed that iso-suillin induced H446 cells G_1 and G_2, cell cycle arrest to decelerate the cell cycle. Further cellular and biochemical analyses indicate that the inhibitory activity of iso-suillin was related to the induction of apoptosis. The cultured H446 cells treated with iso-suillin exhibited typically morphological features of apoptosis [Figure 2]. Flow cytometry tests also indicate that iso-suillin could induce H446 cells apoptosis in a dose- and time-dependent manner. In the present study, we also found that iso-suillin did not affect lymphocyte proliferation at low concentrations but promoted lymphocyte proliferation at higher concentrations. The effects of iso-suillin were specific for cancer cells, and no cytotoxicity in normal lymphocyte was observed. These results suggest that iso-suillin has the potential to be a novel treatment for liver, lung, and gastric cancers.

Apoptosis can be induced through two major pathways: one involving death receptors and the other is cell stress pathway. Cell death receptor-mediated apoptosis is through caspase-8. It is characterized by binding cell death ligands and cell death receptors and subsequently activates application. For example, cisplatin induces apoptosis in cervical cancer SiHa cells.\textsuperscript{13} In these studies, the results from MTT assays indicate that iso-suillin effectively inhibit the growth of H446 cells, BGC-823 cells, SMMC-7721 cells, and MCF-7 cells. Iso-suillin’s inhibition effects in H446 cells (IC_{50}: 9.54 μmol/L) and BGC-823 cells (IC_{50}: 11.60 μmol/L) are close to that of cisplatin (IC_{50}: 14.82 μmol/L). Moreover, iso-suillin inhibited H446 cells growth in a dose- and time-dependent manner. The flow cytometry results also showed that iso-suillin induced H446 cells G_1 and G_2, cell cycle arrest to decelerate the cell cycle. Further cellular and biochemical analyses indicate that the inhibitory activity of iso-suillin was related to the induction of apoptosis. The cultured H446 cells treated with iso-suillin exhibited typically morphological features of apoptosis [Figure 2]. Flow cytometry tests also indicate that iso-suillin could induce H446 cells apoptosis in a dose- and time-dependent manner. In the present study, we also found that iso-suillin did not affect lymphocyte proliferation at low concentrations but promoted lymphocyte proliferation at higher concentrations. The effects of iso-suillin were specific for cancer cells, and no cytotoxicity in normal lymphocyte was observed. These results suggest that iso-suillin has the potential to be a novel treatment for liver, lung, and gastric cancers.

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caspase-8 and caspase-3. The cell stress pathway involves mitochondria-mediated apoptosis through caspase-9. The key element in the pathway is the liberation of the cytochrome c from mitochondria to cytosol. Once cytochrome c is in the cytosol, cytochrome c together with Apaf-1 activates caspase-9, and the latter then activates caspase-3. 

The two pathways converge at caspase-3 activation; thus, caspase-3 is considered a key enzyme in the pathogenesis of cell apoptosis. The level of cleaved caspase-3 represents the level of activated caspase-3. High levels of cleaved caspase-3 in certain tumor may predict good survival. Our results indicate that iso-suillin might increase the cleavage and activity of caspase-3.

The observed decrease in membrane potential suggested the irreversible occurrence of early apoptosis because the permeability of the mitochondrial membrane is increased and apoptotic factors including cytochrome c are released to induce apoptosis. Cytochrome c and caspase-9 are two important proteins involved in the mitochondrial apoptosis pathway. Our results showed that as iso-suillin concentration increased, the MMP of cells gradually decreased. The expression levels of caspase-9 and cytochrome c were increased by iso-suillin, and caspase-9 inhibitor was shown to reverse the apoptosis process in iso-suillin-treated H446 cells.

The release of cytochrome c is deemed to be a process regulated tightly by Bcl-2 family proteins that consist of anti-apoptotic and pro-apoptotic members. It is well known that Bcl-2 functions in preventing apoptosis and can block cell death caused by various stresses such as chemotherapeutic drugs, ultraviolet radiation, free radicals, and withdrawal of growth factors. Bax homodimers, sharing sequence homology with Bcl-2, act as binding proteins to Bcl-2 and favor cell death. In many systems, members of the Bcl-2 family modulate apoptosis, with the ratio of Bax to Bcl-2 serving as a rheostat to determine the susceptibility of cells to apoptosis. In the current study, it was shown that after iso-suillin treatment, Bax expression increased while Bcl-2 expression decreased, and the ratio of Bax/Bcl-2 increased. These results suggest that iso-suillin might activate the mitochondrial pathway and mediates H446 cells apoptosis. FADD binds to death receptors, which in turn activates caspase-8/-3 and could also induce apoptosis. FADD and caspase-8 are two proteins involved in the death receptor pathway of apoptosis. Our results showed that, in treated cells, the expression of FADD and the activation of caspase-8 were up-regulated. Moreover, the caspase-8 inhibitor could reverse the apoptosis process in H446 cells to varying degrees. These results suggest that in H446 cells the apoptosis process induced by iso-suillin is related to the death receptor pathway.

In conclusion, the results of the present study provide evidences that iso-suillin triggers apoptosis through both the death receptor pathway and the mitochondrial pathway; at the same time, also provide a mechanistic framework for further exploration of the use of iso-suillin as a novel chemotherapeutic agent for human lung cancer.

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Conflicts of interest
There are no conflicts of interest.

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