Isoorientin induces the apoptosis and cell cycle arrest of A549 human lung cancer cells via the ROS-regulated MAPK, STAT3 and NF-κB signaling pathways

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Abstract. Isoorientin (ISO) is a naturally occurring C-glycosyl flavone that has various pharmacological properties, such as anti-bacterial and anti-inflammatory effects. However, its underlying molecular mechanisms in human lung cancer cells remain unknown. In the present study, the effects of ISO on the induction of apoptosis and relative molecular mechanisms in A549 human lung cancer cells were investigated. The results of Cell Counting Kit-8 assay (CCK-8) indicated that ISO exerted significant cytotoxic effects on 3 lung cancer cell lines, but had no obvious side-effects on normal cells. Moreover, flow cytometry and western blot analysis revealed that ISO induced mitochondrial-dependent apoptosis by reducing mitochondrial membrane potential. ISO also increased the expression levels of Bax, cleaved-caspase-3 (cle-cas-3) and poly(ADP-ribose) polymerase (PARP; cle-PARP), and decreased the expression levels of Bcl-2 in A549 cells. Furthermore, ISO induced G2/M cell cycle arrest by decreasing the expression levels of cyclin B1 and CDK1/2, and increasing the expression levels of p21 and p27 in A549 cells. As the duration of ISO treatment increased, intracellular reactive oxygen species (ROS) levels in A549 cells also increased. However, pre-treatment of the cells with the ROS scavenger, N-acetylcysteine (NAC), inhibited ISO-induced apoptosis. In addition, ISO increased the expression levels of p-p38, p-JNK and IκB-α; and decreased the expression levels of p-extracellular signal-regulated kinase (ERK), p-signal transducer and activator of transcription (STAT)3, p-nuclear factor (NF)-κB, NF-κB and p-IκB; these effects were induced by mitogen-activated protein kinase (MAPK) inhibitors and blocked by NAC. Taken together, the results of the present study indicate that ISO induces the apoptosis of A549 lung cancer cells via the ROS-mediated MAPK/STAT3/NF-κB signaling pathway, and thus may be a potential drug for use in the treatment of lung cancer.

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Introduction

Lung cancer remains the most prevalent malignant disease and leading cause of mortality worldwide (1). Treatment for the majority of patients with lung cancer includes surgery, chemotherapy, radiation therapy, or a combination of these treatments. Among these, chemotherapy is an effective treatment strategy for lung cancer, which can improve the overall survival rate of patients following surgery. However, side-effects such as nausea, vomiting and drug resistance often limit the use of chemotherapeutic agents (2). Among the chemotherapeutic drugs, naturally occurring flavonoids, such as baicalin, liquiritin, hesperetin and quercetin have garnered substantial interest due to their potential effects on cancer cells and lower toxicity on normal cells (3-6).

Several studies have demonstrated that reactive oxygen species (ROS) are associated with a variety of cellular processes, such as transcription factor activation, cell proliferation and apoptosis (7). The generation of intracellular ROS is an important source of mitochondrial electron transport chains. The excessive generation of intracellular ROS reduces

mitochondrial membrane potential (MMP), leading to apoptosis (8). Moreover, the ROS-mediated mitogen-activated protein kinase (MAPK) pathway is closely associated with cell proliferation, differentiation and apoptosis. Among MAPK families, there is ample evidence that p38/MAPK, c-Jun N-terminal kinases (JNKs) and extracellular signal-regulated kinases (ERKs) are involved on cancer initiation and progression (9). In addition, the ROS-mediated signal transducer and activator of transcription 3 (STAT3) and nuclear factor-κB (NF-κB) pathways play an important role in cancer progression (10-13). Increasing evidence has suggested that ROS induce cell apoptosis by activating the MAPK, STAT3 and NF-κB signaling pathways in cancer cells (14,15).

Isoorientin (3',4',5,7-tetrahydroxy-6-C-glucopyranosyl flavone, ISO) is a well characterized naturally occurring flavonoid with biological properties representative of this group of compounds. It has been extracted from several plant species, including *Patrinia*, *Crataegus pentagyna* and *Drosophyllum lusitanicum* (16-18) and has anti-bacterial and anti-inflammatory activities. It has been demonstrated that ISO inhibits the rate of protein synthesis of *Salmonella typhimurium*, alters the permeability of the cell membrane of the bacteria, and eventually causes the leakage of nucleic acids and electrolytes; it also inhibits the proliferation of bacteria, thereby exerting antibacterial effects (19). In addition, ISO attenuates neuroinflammation by inhibiting the ROS-related MAPK/NF-κB signaling pathway, thereby exerting anti-inflammatory effects (20,21). Furthermore, ISO has been shown to induce apoptosis and cell cycle arrest of HT-29 colorectal adenocarcinoma cells (22). However, the effects of ISO-induced apoptosis on human lung cancer cells remain unknown.

The present study evaluated the anticancer effects of ISO on human lung cancer cells (A549, NCI-H23 and NCI-H460). In addition, ISO-induced apoptosis through the ROS-mediated MAPK, STAT3 and NF-κB signaling pathways in A549 cells was evaluated.

Materials and methods

Cell lines and cell culture. The human lung cancer cell lines A549, NCI-H23 and NCI-H460 cells, were obtained from the American Type Culture Collection (ATCC) and maintained in Dulbecco's modified Eagle's medium (DMEM; Gibco; Thermo Fisher Scientific, Inc.). Normal lung IMR-90 and normal stomach GES-1 cells were obtained from ATCC and Saiqi Biotech Co., Ltd., and maintained in DMEM (Gibco; Thermo Fisher Scientific, Inc.). All cells were supplemented with heat-inactivated fetal bovine serum (FBS; Gibco; Thermo Fisher Scientific, Inc.), 100 μg/ml streptomycin (Gibco; Thermo Fisher Scientific, Inc.) and 100 U/ml penicillin (Gibco; Thermo Fisher Scientific, Inc.). The cells were then cultured at 37˚C in a 5% CO_2 atmosphere.

Cell viability assay. The cytotoxic effects of ISO treatment on lung cancer cells (A549, NCI-H23 and NCI-H460) and the side-effects of ISO (purity ≥99%; Chengdu Herbpurify Co., Ltd.) on normal cells (IMR-90 and GES-1) were assessed using the Cell Counting Kit-8 (CCK-8) assay. Briefly, cells were seeded in 96-well culture plates at a density of $1x10^4$ cells per well and incubated for 24 h at 37˚C. 5-FU, as the one of the first anticancer drug ued, inhibits thymidine nucleotide synthetase, blocks the conversion of deoxypyrimidine nucleotides into thymidine nucleus and interferes with DNA synthesis. In addition, cisplatin can bind to DNA and causes cross-linking, thereby destroying the function of DNA and inhibiting cell mitosis. It is a potent broad-spectrum anticancer drug (23,24). Thus, in the present study, 5-FU and cisplatin were selected as positive control drugs. The lung cancer cells and normal cells were treated with 5-FU (Medchem Express), cisplatin (Solarbio Science & Technology Co., Ltd.) and ISO at various concentrations (20, 40, 60, 80 and 100 μM) for 24 h or different periods of time (3, 6, 12, 24 and 36 h) at 46.81 μM (the IC_{50} value of A549 cells). Subsequently, 10 μl CCK-8 solution were added to each well followed by incubation for 3 h at 37˚C. The absorbance values were measured using a microplate reader (BioTek Instruments Inc.) at 450 nm and the experiments were performed 3 times with 16 wells per experiment. Finally, the obtained OD values were analyzed using GraphPad Prism software, and the corresponding IC_{50} values were then obtained using GraphPad Prism software.

Analysis of cell apoptosis. The effects of ISO on cell apoptosis were measured using the Apoptosis and Necrosis Assay kit and Annexin V Detection kit (Beyotime Institute of Biotechnology). The A549 cells were seeded in 6-well culture plates at a density of $1x10^5$ per well and treated with 46.81 μM ISO for different periods of time (3, 6, 12 and 24 h). After the cells were collected, they were re-suspended in 100 µl cell staining buffer, followed by the addition of 5 μl Hoechst 33342 staining solution and 1.5 μl propidium iodide (PI) staining solution, incubation for 10 min at 37˚C, and observation with a fluorescence microscope (Thermo Fisher Scientific, Inc.). The collected A549 cells were then cultured in 195 μl Annexin V staining buffer, followed by the addition of 3 μl Annexin V-FITC and 2 μl PI for 10 min. The percentages of apoptotic cells were analyzed using a flow cytometry (Beckman Coulter, Inc.).

Detection of MMP. The MMP of A549 cells was detected using the MMP Detection kit (JC-1; Beyotime Institute of Biotechnology). After the A549 cells were grown in a 6-well culture plate at a density of $1x10^5$ cells per well, they were treated with 46.81 μM ISO for different periods of time (3, 6, 12 and 24 h). The A549 cells were incubated with JC-1 working solution at 37˚C for 20 min. The cells were then washed twice with 1X JC-1 staining buffer solution. The data were analyzed by flow cytometry.

Analysis of cell cycle arrest. The cell cycle arrest of ISO-treated A549 cells was assessed by a DNA Content Quantitation assay (Solarbio Science &Technology Co., Ltd.). Briefly, the A549 cells were treated with 46.81 μM ISO for different periods of time (3, 6, 12 and 24 h). After pre-cooling with 70% ethanol overnight, they were washed twice with phosphate-buffered saline (PBS), followed by the addition of 100 μl RNase A solution and the re-suspension of cells at 37˚C for 30 min. Finally, 400 μl PI staining solution were added, and the cells were incubated for 30 min at 4˚C. The data were then analyzed by flow cytometry.

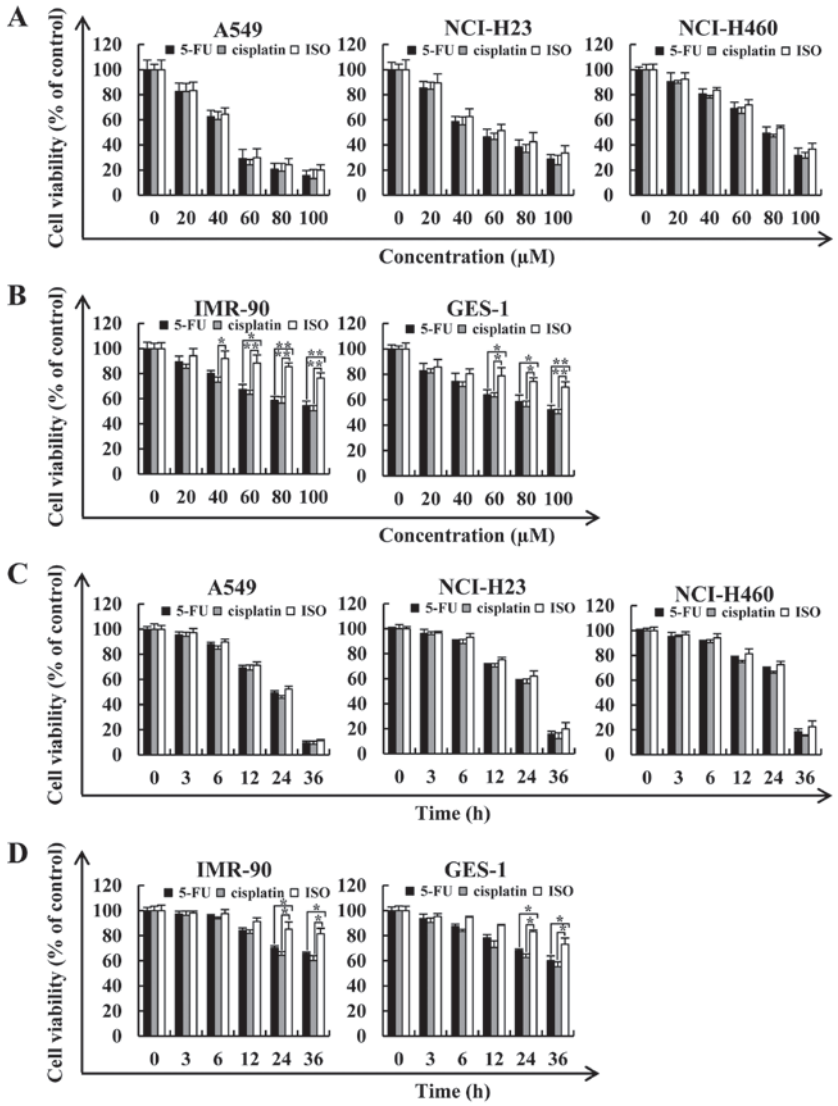

Figure 1. Cytotoxic effects of ISO on human lung cancer cells. (A) A549, NCI-H23 and NCI-H460 lung cancer cells were treated with various concentrations of 5-FU, cisplatin and ISO (20, 40, 60, 80 and 100 μM) for 24 h, after which their cell viabilities were determined by CCK-8 assay. (B) IMR-90 and GES-1 normal cells were treated with various concentrations of 5-FU, cisplatin and ISO (20, 40, 60, 80 and 100 μM) for 24 h, after which their cell viabilities were determined by CCK-8 assay. (C) A549, NCI-H23 and NCI-H460 cells were treated for different periods of time (3, 6, 12, 24 and 36 h) with the IC_{50} value of ISO, after which their cell viabilities were determined by CCK-8 assay. (D) IMR-90 and GES-1 cells were treated for different periods of time (3, 6, 12, 24 and 36 h) with the IC_{50} value of ISO, after which their cell viabilities were determined by CCK-8 assay. Data are expressed as the means ± SD. *P<0.05 and **P<0.01 vs. control. ISO, isoorientin.

Preparation of nuclear extracts. The Nuclear Protein Extraction kit was used to prepare the nuclear extract. The A549 cells were treated with 46.81 μM ISO for different periods of time (3, 6, 12 and 24 h), and then washed with PBS once; the cells were then centrifuged at 500 x g for 3 min in room temperature. After the cells were resuspended with 80 μl plasma protein extraction reagent, they were incubated on ice for 10 min. The cells were then centrifuged at 12,000 x g for 10 min at 4˚C and the supernatants were used as the cytosolic extract. The precipitate was then resuspended in 50 μl nuclear protein extraction reagent and incubated on ice for 10 min. The cells were then centrifuged at 12,000 x g for 10 min at 4˚C. The supernatant was used as the nuclear protein.

Western blot analysis. The expression levels of relevant proteins were measured by western blot analysis. After the A549 cells were treated with 46.81 μM ISO for different periods of times (3, 6, 12 and 24 h), the cells were collected, and protein was extracted using cell lysis buffer. For the inhibitor-treated cell samples, the A549 cells were pre-treated 30 min with 10 μM MAPK inhibitors (the pharmacological inhibitor of p38, SB203580; the pharmacological inhibitor of JNK, SP600125; and the pharmacological inhibitor of ERK, FR180204; all from MedChem Express) at 37˚C and were then treated with 46.81 μM ISO for 24 h. For *N*-acetylcysteine (NAC)-treated cell samples, the A549 cells were pre-treated 30 min with 10 μM NAC (Sigma-Aldrich; Merck KGaA) at 37˚C and then treated with 46.81 μM ISO for 24 h. Briefly, the cells were centrifuged at 12,000 x g for 30 min at 4˚C. An equal amount of protein (30 μg) was loaded onto 10-12% SDS-PAGE gels and electro-transferred onto nitrocellulose membranes (EMD Millipore). The membranes were then blocked in 5% skim milk in Tris-buffered saline Tween-20 (TBST) for 2 h, followed by overnight incubation at 4˚C with specific primary antibodies

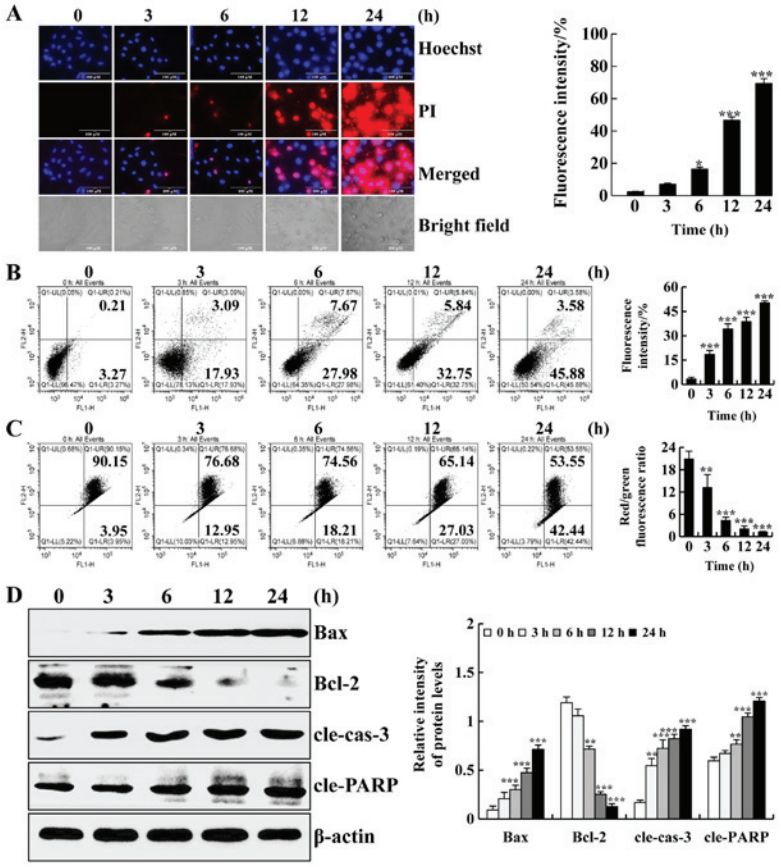

Figure 2. Apoptotic effects of ISO on A549 cells. (A) A549 cells were stained with Hoechst 33342 and PI, and the fluorescence intensities and morphology changes of cells were observed under a fluorescence microscope (original magnification, x200). (B) Percentages of apoptotic cells were detected by flow cytometry. (C) MMP was detected by flow cytometry. (D) The protein expression levels were measured by western blot analysis following treatment of A549 cells with ISO. The percentages of apoptotic cells represent the mean ± SD; β-actin was used as the loading control. *P<0.05, **P<0.01 and ***P<0.001 vs. 0 h. ISO, isoorientin; MMP, mitochondrial membrane potential.

(from Santa Cruz Biotechnology, Inc.), against mouse monoclonal β-actin (1:2,500; cat. no. sc-47778), Lamin B1 (1:2,500; cat. no. sc-374015), Bax (1:1,500; cat. no. sc-493), Bcl-2 (1:1,500; cat. no. sc-7382), cleaved-caspase-3 (cle-cas-3; 1:1,500; cat. no. sc-373730), poly(ADP) ribose polymerase (PARP)-1 (1:1,500; cat. no. sc-8007), p-p38 (1:1,500; cat. no. sc-7973), p-JNK (1:1,500; cat. no. sc-6254), JNK (1:1,500; cat. no. sc-7345), p-ERK (1:1,500; cat. no. sc-7383), p-STAT3 (1:1,500; cat. no. sc-8059), STAT3 (1:1,500; cat. no. sc-8019), NF-κB (1:1,500; cat. no. sc-8008), p-NF-κB (1:1,500; cat. no. sc-166748), inhibitor of IκB-α (IκB-α; 1:1,500; cat. no. sc-1643), p-IκB-α (1:1,500; cat. no. sc-8404), cyclin B1 (1:1,500; cat. no. sc-245), CDK1/2 (1:1,500; cat. no. sc-53219), against rabbit monoclonal p38α/β (1:1,500; cat. no. sc-7149), p27 (1:1,500; cat. no. sc-528), p21 (1:1,500; cat. no. sc-397). The membranes were then incubated with horseradish peroxidase (HRP)-conjugated secondary antibodies (i.e., HRP-conjugated AffiniPure goat anti-mouse IgG and HRP-conjugated AffiniPure goat anti-rabbit IgG) for 1 h at room temperature. Proteins were measured using enhanced chemiluminescence kits (Bio-Rad Laboratories, Inc.). Band intensity was assessed using ImagesJ software version 1.42q.

Detection of intracellular ROS levels. Intracellular ROS levels were measured using the 2',7'-dichlorofluorescein diacetate (DCFH-DA; Beyotime Institute of Biotechnology) fluorescent probe. The cells were treated with 46.81 μM ISO for different periods of time (3, 6, 12 and 24 h). The collected cells were washed twice with PBS. The cells were then incubated with DCFH-DA for 30 min at 37˚C, and were again washed twice with PBS. The fluorescence intensity of DCF, which represents intracellular ROS levels, was analyzed using a flow cytometer (Beckman Coulter, Inc.) in the cell samples.

Statistical analysis. All data are presented as the means ± standard deviation from 3 experiments. Continuous data were analyzed by one-way analysis of variance followed by Tukey's post-hoc test using SPSS software version 21.0. P-values <0.05 were considered to indicate statistically significant differences.

Results

ISO exerts cytotoxic effects on human lung cancer cells. To determine the effects of ISO on lung cancer cell viability, the A549, NCI-H23 and NCI-H460 cells were treated with 5-FU and ISO, and after 24 h, cell viability was measured by CCK-8 assay. As shown in Fig. 1A, 5-FU, cisplatin and ISO exerted significant cytotoxic effects on lung cancer cells (A549, NCI-H23 and NCI-H460) in a dose-dependent manner. As human lung fibroblasts (IMR-90) are extracted from embryonic cells, their primary properties have not transformed. This can directly reflect the toxic effects to the human lungs in the

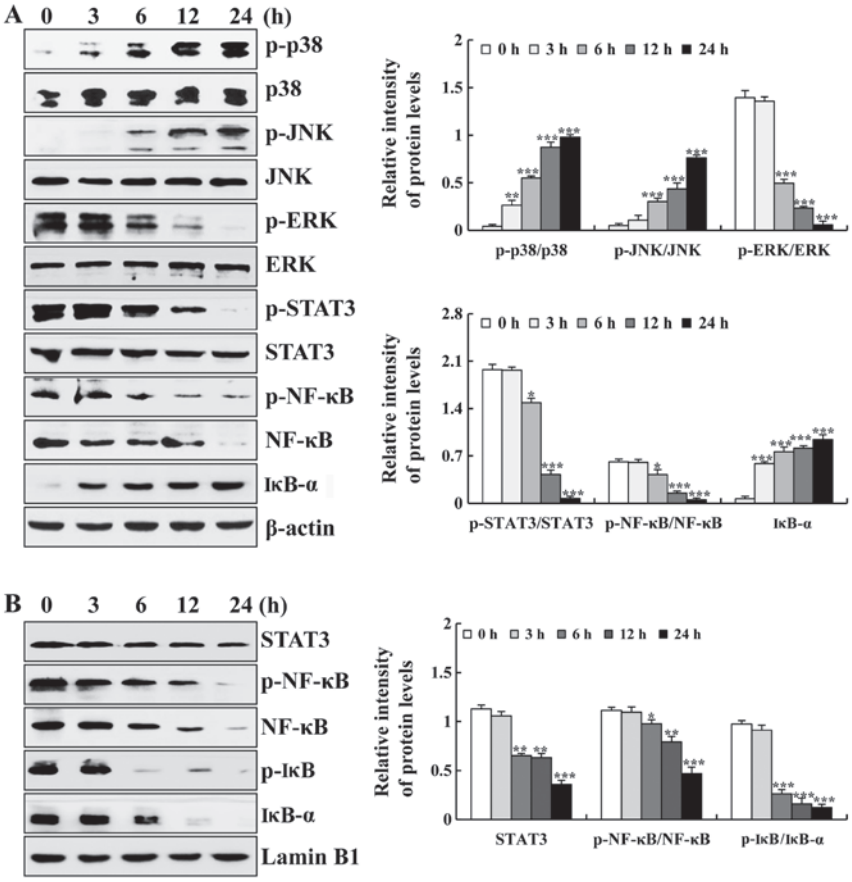

Figure 3. Effects of ISO on MAPK/STAT3/NF-κB signaling pathway in A549 cells. (A) Expression levels of p-p38, p-JNK, p-ERK, p-STAT3, p-NF-κB, NF-κB and IκB-α were measured by western blot analysis. (B) Expression levels of STAT3, p-NF-κB, NF-κB, p-IκB and IκB-α in the nucleus were measured by western blot analysis. The phosphorylated proteins were quantified with corresponding total proteins. β-actin and Lamin B1 were used as the loading controls. *P<0.05, **P<0.01 and ***P<0.001 vs. 0 h. ISO, isoorientin.

toxicity experiment of the compounds. Furthermore, GES-1 cells are human gastric mucosal epithelial cells. The drug used in this experiment is a compound extracted from Chinese herbal medicine, which is mainly digested and metabolized in the stomach. Therefore, the IMR-90 and GES-1 cells were selected for the determination of ISO cytotoxicity. Importantly, compared with 5-FU and cisplatin, following treatment with ISO, the survival rate of the human normal cells (IMR-90 and GES-1) did not evidently decrease (Fig. 1B). Among the lung cancer cells, the A549 cells were more sensitive to ISO than the NCI-H23 and NCI-H460 cells, and the half-maximal inhibitory values (IC_{50}) of ISO for the lung cancer cells are presented in Table I. In addition, the results of CCK-8 assay revealed that 5-FU, cisplatin and ISO exerted evident growth-inhibitory effects on the lung cancer cells and the IC_{50} value was reached in the A549 cells at 24 h (Fig. 1C). It was found that the concentration of ISO did not reach its IC_{50} value when it exceeded 100 μM; thus, it was considered that its IC_{50} value exceeds 100 μM. In addition, in order to ensure that normal cells and cancer cells were compared at the same level, the IC_{50} value of ISO for the experiments was selected based on the toxicity of normal cell time gradients. As shown in Fig. 1D, compared with 5-FU and cisplatin, treatment with 46.81 μM ISO for 24 h exerted no obvious effects on normal cells (Fig. 1D). Based on the results shown in Fig. 1C and D, it was found that the cytotoxic effects of ISO on lung cancer cells were similar to those of 5-FU and cisplatin; however, ISO exerted less side-effects than 5-FU and cisplatin on normal cells. In addition, when the cells were treated with 46.81 μM ISO for different periods of time (3, 6, 12, 24 and 36 h), the cells begun to undergo apoptosis and gradually die. In particular, when the treatment time reached 36 h, a large number of cells were apoptotic and exfoliated from the plate, rendering subsequent experiments impossible. Thus, subsequent experiments were conducted at different time points (3, 6, 12 and 24 h).

ISO induces the apoptosis of A549 cells. To verify the effects of ISO on lung cancer cell apoptosis, the A549 cells were processed with ISO for different periods of time (3, 6, 12 and 24 h), and the fluorescence intensity was detected with a fluorescence microscope. As shown in Fig. 2A, the fluorescence intensity of Hoechst 33342 and PI and the degree of cell shrinkage were increased. Next, we detected the apoptotic effects of ISO in A549 cells by flow cytometry. As shown in Fig. 2B, the ratio of apoptotic cells was increased. In addition, early apoptotic cells were accompanied by changes in MMP. As shown in Fig. 2C, the ratio of red to green fluorescence was significantly decreased, indicating that ISO reduced MMP in a time-dependent manner. Consistently, to further investigate the molecular mechanisms through which ISO induced the apoptosis of A549 cells, the expression levels of apoptosis-related proteins were examined by western blot analysis. As shown in

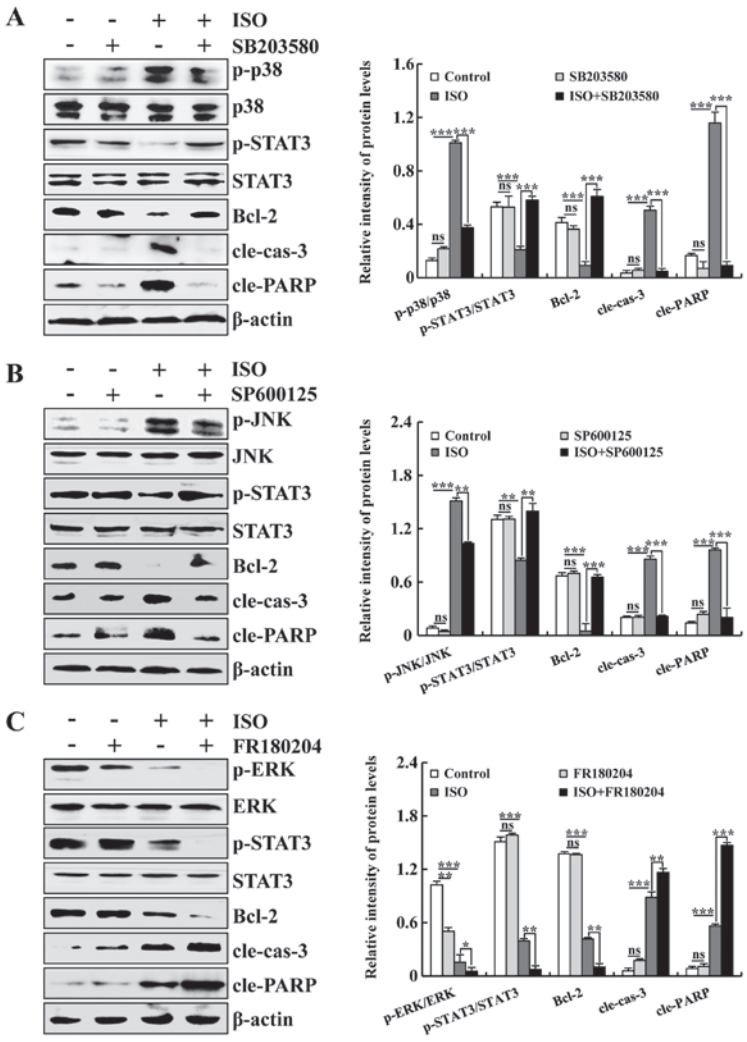

Figure 4. Effects of ISO on the MAPK and STAT3 signaling pathways in A549 cells. (A) Expression levels of p-p38, p-STAT3, Bcl-2, cle-cas-3 and cle-PARP proteins in ISO-treated and p38 inhibitor-treated A549 cells. (B) Expression levels of p-JNK, p-STAT3, Bcl-2, cle-cas-3 and cle-PARP proteins in ISO-treated and JNK inhibitor-treated A549 cells. (C) Expression levels of p-ERK, p-STAT3, Bcl-2, cle-cas-3 and cle-PARP proteins in ISO-treated and ERK inhibitor-treated A549 cells. The phosphorylated proteins were quantified with corresponding total proteins. β-actin was used as the loading control. *P<0.05, **P<0.01 and ***P<0.001 vs. ISO + MAPK inhibition. ns, not significant; ISO, isoorientin.

Fig. 2D, the protein expression levels of Bax, cleaved caspase-3 (cle-cas-3) and cleaved PARP (cle-PARP) were increased. Furthermore, the protein expression levels of Bcl-2 were decreased. Thus, these results demonstrated that ISO induced apoptosis via a mitochondrial-dependent pathway in A549 cells.

ISO induces apoptosis through the MAPK, STAT3 and NF-κB signaling pathways in A549 cells. To further determine the molecular mechanisms responsible for the ISO-induced apoptosis of A549 cells, the related protein expression levels of MAPK, STAT3 and NF-κB were measured by western blot analysis. As shown in Fig. 3A, the protein expression levels of p-p38, p-JNK and IκB-α were increased, whereas the protein expression levels of p-ERK, p-STAT3, p-NF-κB and NF-κB were decreased. As a nuclear transcription factor, the nuclear translocation of proteins (STAT3, p-NF-κB, NF-κB, p-IκB and IκB-α) is required for their function. As shown in Fig. 3B, the nuclear protein expression levels of STAT3, p-NF-κB, NF-κB, p-IκB and IκB-α were decreased. In addition, to verify the effects of MAPK and STAT3 signaling pathways on the ISO-induced apoptosis of human lung cancer cells, the A549 lung cancer cells were pre-treated with 10 μM SB203580 (a pharmacological inhibitor of p38), 10 μM SP600125 (a pharmacological inhibitor of JNK) and 10 μM FR180204 (a pharmacological inhibitor of ERK), and the protein expression levels of the MAPKs and STAT3 were measured by western blot analysis. As shown in Fig. 4A and B, following pre-treatment with SB203580 or SP600125 alone, no significant differences were observed between the SB203580 group or SP600125 group and the control group. Compared with the control group, the protein expression levels of p-STAT3 and Bcl-2 were decreased, while the protein expression levels of p-p38, p-JNK, cle-cas-3 and cle-PARP were increased in the ISO group. Moreover, following pre-treatment with SB203580 or SP600125, compared with the ISO group, the protein expression levels of p-STAT3 and Bcl-2 were increased, while the protein expression levels of p-p38, p-JNK, cle-cas-3 and cle-PARP were decreased in the ISO + SB203580 group

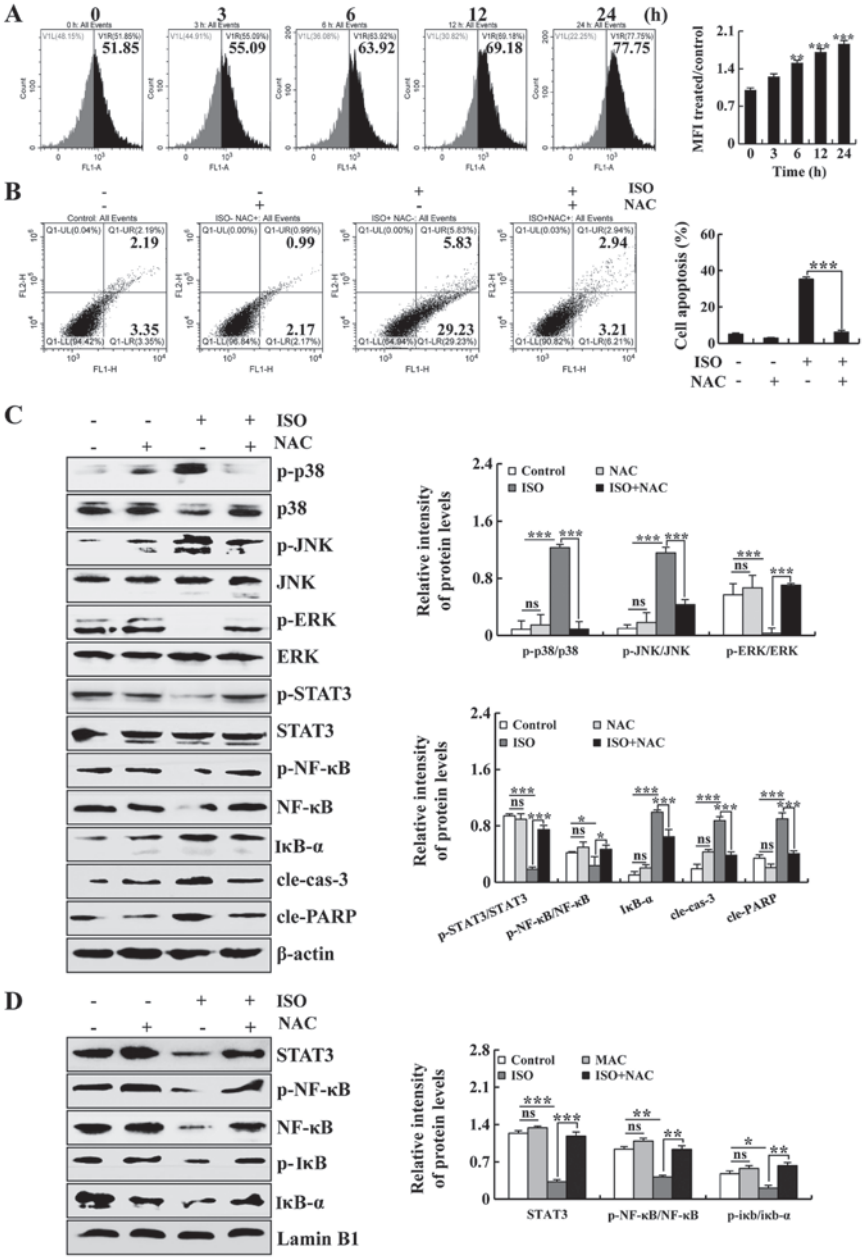

Figure 5. Promoting effects of ISO on ROS generation and the induction of apoptosis of A549 cells. (A) A549 cells were treated with ISO, and the intracellular ROS levels were measured by flow cytometry. (B) A549 cells were treated with NAC and ISO. The percentages of apoptotic cells were then measured by flow cytometry. (C) A549 cells were treated with ISO and NAC, after which the expression levels of MAPKs, STAT3, NF-κB, cle-cas-3 and cle-PARP were detected by western blot analysis. (D) A549 cells were treated with ISO and NAC, after which the expression levels of STAT3, p-NF-κB, NF-κB, p-iκb and iκb-α in the nucleus were detected by western blot analysis. The phosphorylated proteins were quantified with corresponding total proteins. β-actin and Lamin B1 was used as the loading controls. *P<0.05, **P<0.01 and ***P<0.001 vs. the NAC + ISO group. ns, not significant; ISO, isoorientin.

or ISO + SP600125 group. As shown in Fig. 4C, following pre-treatment with FR180204 alone, the expression level of p-ERK was decreased and that of the other 4 proteins exhibit no significant difference between the FR180204 group and the control group. Briefly, compared with the control group, the protein expression levels of p-ERK, p-STAT3 and Bcl-2 were decreased, while the protein expression levels of cle-cas-3 and cle-PARP were increased in the ISO group. Furthermore, following pre-treatment with FR180204, compared with the ISO group, the protein expression levels of p-ERK, p-STAT3 and Bcl-2 were decreased, while the protein expression levels of cle-cas-3 and cle-PARP were increased in the ISO + FR180204 group. Taken together, these results indicated that the MAPK/STAT3/NF-κB signaling pathways were associated with the ISO-induced apoptosis of A549 cells.

ISO-induced cell apoptosis is dependent on the ROS-mediated MAPK/STAT3/NF-κB signaling pathway in A549 cells. To investigate whether ROS are associated with the ISO-induced apoptosis of A549 cells, intracellular ROS levels were detected by flow cytometry. As shown in Fig. 5A, following treatment with ISO, the intracellular ROS levels were significantly increased. In addition, the apoptosis of A549 cells was significantly decreased following pre-treatment with NAC (Fig. 5B). To further investigate whether ISO induces cell apoptosis

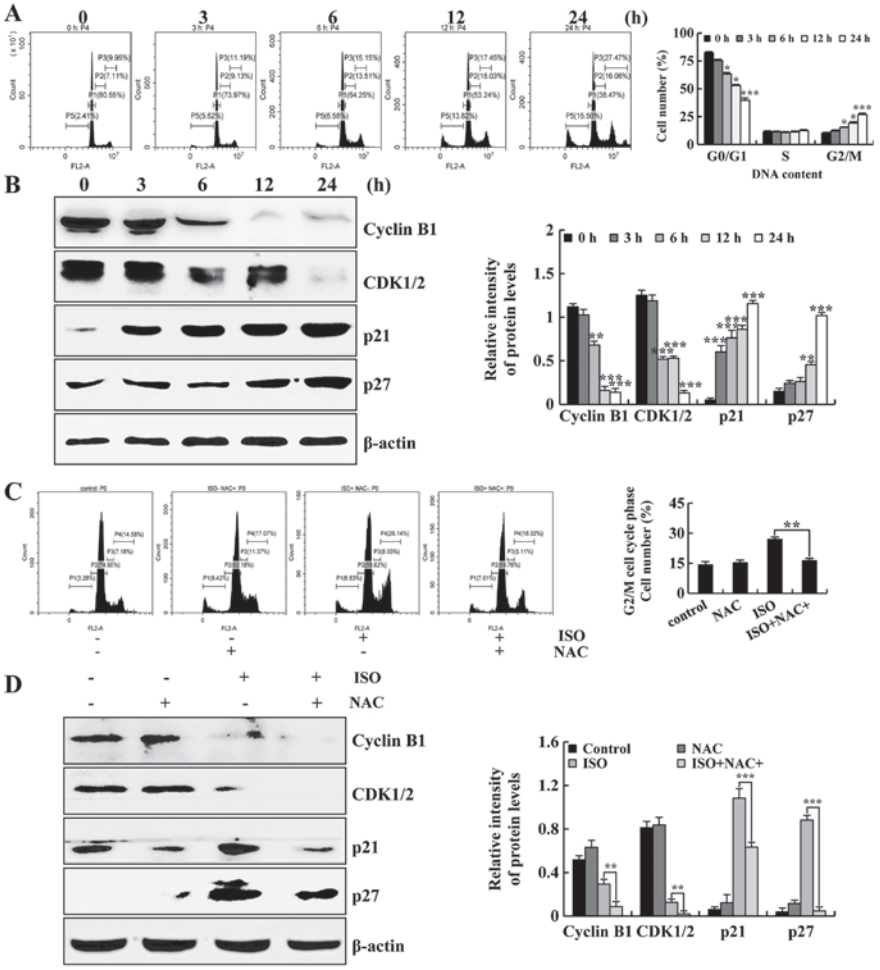

Figure 6. Effects of ISO on the cell cycle of A549 cells. (A) The percentages of cell cycle were detected by flow cytometry. (B) Expression levels of G2/M cell cycle-related proteins cyclin B1, CDK1/2, p21 and p27 were examined by western blot analysis following treatment with ISO. (C) A549 cells were treated with NAC and ISO, and the percentages of cell cycle were then detected by flow cytometry. (D) A549 cells were treated with ISO and NAC, after which the expression levels of cyclin B1, CDK1/2, p21, and p27 were detected by western blot analysis. β-actin was used as the loading controls. *P<0.05, **P<0.01 and ***P<0.001 vs. 0 h or the NAC + ISO group. ISO, isoorientin.

via the ROS-mediated MAPK, STAT3 and NF-κB signaling pathways, the A549 cells were pre-treated with NAC, and the protein expression levels of MAPK, STAT3, p-NF-κB and NF-κB, as well as the nuclear protein expression levels of STAT3, p-NF-κB, NF-κB, p-IκB and IκB-α were then detected. As shown in Fig. 5C, following pre-treatment with NAC, no significant differences were observed between the NAC group and the control group. Compared with the control group, the expression levels of p-p38, p-JNK, IκB-α, cle-cas-3 and cle-PARP were significantly upregulated, while the expression levels of p-ERK, p-STAT3, p-NF-κB and NF-κB were downregulated in the ISO group. Furthermore, following pre-treatment with NAC, compared with the ISO group, the expression levels of p-p38, p-JNK, IκB-α, cle-cas-3 and cle-PARP were significantly downregulated, while the expression levels of p-ERK, p-STAT3, p-NF-κB and NF-κB were upregulated in the ISO + NAC group. In addition, as shown in Fig. 5D, following pre-treatment with NAC, no significant differences were observed between the NAC group and the control group. Compared with the control group, the nuclear protein expression levels of STAT3, p-NF-κB and NF-κB were significantly decreased in the ISO group. In brief, following pre-treatment with NAC, compared with the ISO group, the nuclear protein expression levels of STAT3, p-NF-κB, NF-κB, p-IκB and IκB-α were increased in the ISO + NAC group.

ISO leads to the G2/M cell cycle arrest of the A549 cells. To determine the effects of ISO on lung cancer cell cycle arrest, the A549 cells were treated with ISO for different periods of time (3, 6, 12 and 24 h) followed by detection with flow cytometry. As shown in Fig. 6A, the population of A549 cells in the G2/M phase was markedly increased. Subsequently, the expression of G2/M cell cycle-related proteins was detected by western blot analysis. It was found that the protein expression levels of cyclin B1 and CDK1/2 were decreased. Moreover, the protein expression levels of p21 and p27 were increased. These data indicated that ISO caused the G2/M cell cycle arrest of the A549 cells. Furthermore, the cells were pre-treated with NAC, and the population of A549 cells in the G2/M phase was measured by flow cytometry. As shown in Fig. 6C, compared with the ISO group, the population of G2/M phase cells was significantly decreased in the ISO + NAC group. Moreover, NAC significantly downregulated the expression levels of cyclin B1 and CDK1/2, and upregulated the expression levels of p21 and p27 compared to ISO treatment alone (Fig. 6D). Taken together, these results suggested that ISO induced

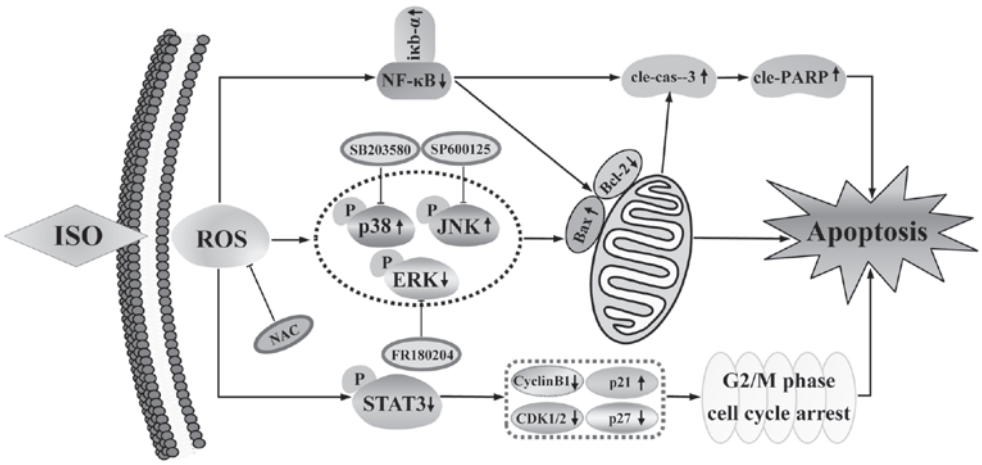

Figure 7. The underlying mechanisms of the anticancer activity of ISO on A549 human lung cancer cells. ISO, isoorientin.

Table I. IC_{50} values of ISO and 5-FU in lung cancer cells.

Cell line	5-FU (μM)	ISO (μM)
A549	43.52±1.83	46.81±2.37
NCI-H23	57.15±2.14	63.88±1.49
NCI-H460	79.82±1.54	81.69±1.56

The IC_{50} values were calculated using GraphPad Prism software.

apoptosis and G2/M cell cycle arrest via the ROS-mediated MAPKs/STAT3/NF-κB signaling pathways in A549 human lung cancer cells (Fig. 7).

Discussion

Traditional Chinese herbal medicine is one of the topics in the antitumor drug development arena. Certain studies have noted that herbs with anticancer effects can be separated into several compounds including flavonoids (25,26). ISO, a naturally C-glycosyl flavone, has been shown to inhibit the proliferation of HepG2 liver cancer cells, with no obvious cytotoxicity on HL-7702 normal human liver cells (27). Several studies have demonstrated that the anticancer mechanisms of ISO involve the inhibition of cancer cell proliferation, the promotion of oxidative stress, the induction of cell cycle arrest, and ultimately, in the induction of apoptosis (22,27-34). However, the experimental data of the present study revealed that ISO induced ROS accumulation, apoptosis and G2/M cycle arrest in the lung cancer cells, and the present study also investigated the relevant molecular mechanisms. It was found that ROS accumulation induced by ISO activated the MAPK signaling pathway and further inhibited the STAT3 and NF-κB signaling pathways. ISO also exerted effects on nuclear transcription factors, such as the nuclear translocation of proteins (STAT3, p-NF-κB, NF-κB and p-IκB), finally causing the apoptosis of A549 lung cancer cells. On the other hand, ROS accumulation regulated the expression of CDK and cyclin, causing the G2/M phase arrest of the A549 lung cancer cells. Finally, a theoretical basis was provide for the drug design and anticancer drug development by ISO. It has previously found that the IC_{50} value of the HT-29 cells was 125 μM, the IC_{50} of HepG2 cells was 80 μM, the IC_{50} of PATU-8988 cells was 300 μM, and the IC_{50} of PANC-1 cells was 120 μM (22,27,29,31). The present study demonstrated that ISO decreased A549 cell viability in dose-dependent and time-dependent manner; moreover, no evident side-effects were observed on the normal cells in the 5-FU and cisplatin group; the IC_{50} value of the A549 cells was 46.81 μM, the IC_{50} of the NCI-H23 cells was 63.88 μM and the IC_{50} of the NCI-H460 cells was 81.69 μM (Fig. 1 and Table I). In addition, following treatment with ISO for 24 h, the A549 cells exfoliated from the plate and exhibited morphology similar to apoptosis (Fig. 2A) (35). To further define the underlying mechanisms of ISO, the effects of ISO on the induction of apoptosis of the A549 cells were then investigated.

Apoptosis plays a crucial role in the process of cell proliferation, differentiation and death. Mitochondrial-dependent apoptosis is an intrinsic apoptosis, causing the release of cytochrome c, which results in caspase-3 cleavage and ultimately leads to apoptosis (36). Bcl-2 family members are major regulators of the mitochondrial release of cytochrome c and can alter their conformation and form mitochondrial permeability transition pores in the mitochondrial outer membrane, releasing cytochrome c from the mitochondria into the cytosol (37,38). In addition, previous studies have demonstrated that Bcl-2 and Bax control programmed cell apoptosis (39,40). The results of the present study revealed that ISO significantly inhibited the expression levels of Bcl-2 and increased the expression of Bax, cle-cas-3 and cle-PARP (Fig. 2D). Therefore, ISO-induced apoptosis is associated with mitochondrial-dependent pathways.

The activation of the MAPK signaling pathway plays a key role in the apoptosis of cancer cells induced by natural compounds (41,42). Accumulating evidence has identified that the MAPK, STAT3 and NF-κB signaling pathways play important roles in cell apoptosis (43,44). The results of the present study demonstrated that ISO activated the p38 and JNK pathways, inhibiting the ERK, STAT3 and NF-κB signaling pathways. In addition, the expression levels of p-STAT3 were decreased following the addition of p38 and JNK inhibitors (10 μM) and were increased following the addition of an ERK inhibitor (10 μM), indicating that MAPK was involved in

the regulation of the STAT3 signaling pathway in A549 lung cancer cells (Fig. 4) (32,45-48).

A number of studies have demonstrated that some natural flavonoids appear as pro-oxidants or antioxidants depending upon the target cell (49,50). The previous studies have demonstrated that ISO can upregulate ROS levels in HepG2 hepatocellular carcinoma cells to induce cell apoptosis (20). The results revealed that ISO upregulated the levels of ROS. In addition, following treatment of A549 cells with NAC the number of apoptotic cells was significantly reduced (Fig. 5B). It has been reported that ROS can induce apoptosis as a second messenger of MAPK and NF-κB transcription factors in cancer cells. NF-κB factor is localized in the cytoplasm and is sequestered by IκB molecules. Under some stimulating conditions, IκB undergoes ubiquitination and degradation, resulting in the translocation of NF-κB dimers into the nucleus (51-53). In the present study, it was found that ROS was involved in ISO-induced MAPK/STAT3/NF-κB signaling pathways as an upstream signal. Furthermore, NAC regulated the activation of the MAPK, STAT3 and NF-κB signaling pathways (Fig. 5C). The results also revealed that ISO decreased the expression levels of STAT3, p-NF-κB, NF-κB, p-IκB and IκB-α in the nucleus (Fig. 5D). These results indicated that ROS activated the MAPK, STAT3 and NF-κB signaling pathways on the ISO-induced apoptosis of A549 cells.

The balance of the cell cycle is critical for maintaining intracellular stability; however, when cells are damaged, the cell cycle is arrested by various mechanisms, such as the expression of CDKs and the inhibition of cyclins (54-56). In the present study, the results of flow cytometry revealed that the number of cells in the G2/M phase increased following ISO treatment and the number of cells in the G0/G1 phase decreased. Western blot analysis revealed that G2/M phase-associated protein expression levels of CDK1/2 and cyclin B1 decreased following ISO treatment. In addition, the expression levels of p21 and p27 proteins were increased. To summarize, ISO induced the cell cycle arrest of A549 lung cancer cells in the G2/M phase.

In conclusion, the present study demonstrated that, ISO upregulated intracellular ROS levels, caused G2/M cell cycle arrest, and induced apoptosis by regulating the MAPK/STAT3/NF-κB signaling pathway in A549 human lung cancer cells. These results demonstrate the possibility of ISO as a potential treatment agent for lung cancer.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

CHJ and DJZ conceived and designed the experiments. WTX and GNS wrote the manuscript and participated in the experiments. TZL and YZ assessed the cytotoxic effects of the drugs. TZ and HX performed the cell cycle and apoptotic analyses. WBZ performed the western blot analysis. YNL performed the signaling analysis. All authors read and approved the final version of the manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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