Original Article
Use of Saikosaponin D and JNK inhibitor SP600125, alone or in combination, inhibits malignant properties of human osteosarcoma U2 cells

Tian Gao1*, Ping Zhao2*, Xiaolong Yu1*, Suixia Cao2, Bin Zhang1, Min Dai1

1Department of Orthopedics, Artificial Joints Engineering and Technology Research Center of Jiangxi Province, The First Affiliated Hospital of Nanchang University, Nanchang 330006, P. R. China; 2Medical School of Nanchang University, Nanchang 330006, P. R. China. *Equal contributors.

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Abstract: Saikosaponin D (Ssd) is a major active ingredient derived from the traditional Chinese medicinal herb Bupleurum falcatum, and SP600125 is a specific inhibitor of JNK that competes with adenosine triphosphate. In this study, we co-analyzed cell proliferation, apoptosis, migration, and invasion in U-2OS osteosarcoma cells treated with Ssd and SP600125 alone or in combination. Cell death and signaling were analyzed using western blotting and flow cytometry. We observed dramatic inhibition of cellular proliferation, invasion, and migration in cells treated with Ssd alone or in combination with SP600125. Ssd, alone or in combination with SP600125, enhanced Cytochrome C release, increased the Bax/Bcl-2 ratio, and activated caspase-3, -8 and -9, indicating that cellular apoptosis was induced via both the mitochondrial and death receptor pathways. The effect of SP600125 alone on U2 cells was not significant. Additional evaluation of Mcl-1, Akt, p-Akt, ERK, and p-ERK supported an anti-tumor effect of Ssd, which was enhanced in combination with SP600125. This study provides a theoretical basis for the treatment of osteosarcoma with Ssd alone or in combination with SP600125.

Keywords: Osteosarcoma, saikosaponin D, SP600125, cell culture, anti-cancer

Introduction

Although the incidence of human osteosarcoma is low, its prognosis is often poor when compared to other cancer types. Distant metastasis and recurrence are major challenges in the treatment of osteosarcoma, and novel drugs that can inhibit tumor migration and invasion are urgently required [1, 2]. Saikosaponin D (Ssd) is one of the key active components of the Chinese medicine Bupleurum. Studies have confirmed that Ssd has multiple biological activities such as anti-inflammatory, immunoregulatory, anti-fibrosis, and tumor-inhibiting activities [3-5]. Studies focusing on the anti-cancer mechanism of Ssd showed that the compound can initiate apoptosis of tumor cells by activating caspase-3, inhibit cell proliferation, and regulate alterations in cell morphology by inducing tumor cell differentiation. Additionally, studies have shown that Ssd can significantly promote the release of calcium ions in tumor cells, interfere with protein biosynthesis, and thus inhibit cell division and proliferation [6]. However, detailed studies on the effects of Ssd on osteosarcoma are rare.

SP600125, discovered in 2001, is a JNK-specific inhibitor competitive with adenosine triphosphate that blocks the JNK catalytic region of all subtypes and reversibly inhibits JNK activity [7]. There is currently no study of the effect of JNK inhibitor SP600125 on osteosarcoma.

Apoptosis consists of two signaling pathways: exogenous or death receptor pathways regulated by the binding of extracellular ligands of the tumor necrosis factor (TNF) family to death receptors, and intrinsic or mitochondrial pathways controlled by the Bcl-2 family (i.e., Bcl-w, Mcl-1, Bax, Bak, Bid, Noxa, Puma, and Bim) [8]. Some studies have shown that JNK inhibitor SP600125 combined with dihydroartemisinin can enhance apoptosis of human lung adenocarcinoma cells [9]. In this study, we evaluat-
Saikosaponin D and SP600125 inhibits malignant properties of U2 cells

Materials and methods

Cell culture

The human osteosarcoma cell line U-2OS was obtained from the Central Culture Collection of the Chinese Academy of Sciences (Shanghai, China) and supplemented with 10% (v/v) FBS, 100 U/ml penicillin, and 100 μg/ml streptomycin. The cells were grown at 37°C in humidified air with 5% CO₂. The passage number for cells used in experiments was less than 20, and cells were always used in exponential phase.

Materials

Ssd (> 99% purity, HPLC) and SP600125 (inhibitor of JNK that competes with adenosine triphosphate) were purchased from Shanghai Yuanye Biological Technology (Shanghai, China). Stock solutions of Ssd and SP600125 were prepared in DMSO (Sigma, St Louis, MO, USA), and an equal volume of DMSO was added to the controls. All other chemicals were purchased from Sigma-Aldrich unless otherwise stated.

Wound healing analysis for cell migration

U-2OS cells were seeded in 6-well culture plates to reach a confluence greater than 90% on the second day, when a scratch wound was introduced with a sterile 100-μl pipette tip followed by a PBS wash to remove the isolated cells. The injured U-2OS monolayer was immediately treated with Ssd and SP600125 (0 μM Ssd/2 μl DMSO; 5 μM, 10 μM, and 20 μM Ssd; 20 μM SP600125; 5 μM Ssd + 20 μM SP600125; 10 μM Ssd + 20 μM SP600125; 20 μM Ssd + 20 μM SP600125) and incubated for an additional 12, 24, or 36 hours. The wound area was imaged by an inverted microscope (original magnification, 100 ×), the mobility of cells treated with 0 μM Ssd was set to 1.0, and the proportion of migrated cells was calculated as a percentage of the control.

Cell viability determination by CCK-8 method

U-2OS cells were cultured in 96-well plates (5 × 10³ cells/well). Cells were treated with Ssd and SP600125 (0 μM Ssd/2 μl DMSO; 10 μM Ssd; 20 μM Ssd; 20 μM SP600125; 10 μM Ssd + 20 μM SP600125; 20 μM Ssd + 20 μM SP600125) for 24, 36, and 48 hours. After the indicated incubation time, 10 μl of CCK-8 was added to the plates, which were incubated for an additional 1-4 hours at 37°C. Thereafter, the absorbance was measured at 450 nm using an ELISA plate reader (Model EXL800; BioTek Instruments, Inc., Winooski, VT, USA).

Hoechst 33258 staining for observation of nuclei

Cells were incubated with Ssd and SP600125 (0 μM Ssd/2 μl DMSO; 20 μM Ssd; 20 μM SP600125; 20 μM Ssd + 20 μM SP600125) in 6-well plates for 24 hours. Drug-treated cells were fixed with 4% paraformaldehyde for 30 minutes followed by three washes with pre-cooled PBS and stained with 10 mg/l Hoechst 33258 solution for 10 minutes at 25°C in the dark. Subsequently, the stained nuclei were observed under a fluorescence microscope (Olympus Corp., Tokyo, Japan) at 350 nm excitation and 460 nm emission (original magnification, 100 ×).

Analysis of apoptosis by annexin V-FITC/PI staining assay

U-2OS cells were incubated with Ssd and SP600125 (0 μM Ssd/2 μl DMSO; 20 μM Ssd; 20 μM SP600125; 20 μM Ssd + 20 μM SP600125), washed twice with ice-cold PBS, and resuspended at 1 × 10⁶ cells/ml in binding buffer. The cell suspension (100 μl) was incubated with 1 μl of Annexin V-FITC and 2 μl of PI solution for 15 minutes at 25°C in the dark. After addition of 150 μL of 1 × binding buffer, samples were analyzed on a FACSVerse™ flow cytometer (BD Biosciences, San Jose, CA, USA). Apoptosis rates were analyzed using FlowJo 7.6 software (Tree Star, Inc., Ashland, OR, USA).

Cell invasion assay

Transwell cell culture insert membranes (8-μm pore size) were coated with 100 μg/ml Matrigel, followed by seeding 1 × 10⁶ cells in the upper chamber and incubation with Ssd and SP600125 (0 μM Ssd/2 μl DMSO; 20 μM Ssd; 20 μM SP600125; 20 μM Ssd + 20 μM SP600125). The lower wells were filled with RPMI-1640 medium supplemented with 20% FBS.
Saikosaponin D and SP600125 inhibits malignant properties of U2 cells

(v/v) FBS in 24-well plates. After 24 hours of incubation, the non-invasive cells in the upper chamber were removed with a cotton swab, and cells attached to the lower surface were fixed with pre-cooled methanol and stained with crystal violet solution (0.1%). Five areas of each chamber were randomly selected, and the number of cells was determined under the microscope (original magnification, 100 ×).

Western blot analysis

Protein lysates were prepared from U-2OS cells and quantified by BCA assay according to the instructions. Antibodies against Akt, p-Akt, ERK1/2, p-ERK1/2, Mcl-1, Bax, Bcl-2, Cytochrome C, caspase-3, caspase-8, caspase-9, PARP, and GAPDH were purchased from Abcam (Cambridge, UK). Horseradish peroxidase (HRP) coupled secondary antibody was purchased from TransGen Biotech (Beijing, China). GAPDH was used as an internal control for western blotting. Protein levels were normalized to GAPDH levels in three independent experiments and were relatively quantified. Bands were detected using an enhanced chemiluminescence (ECL) detection system from Amersham Pharmacia Biotech (Arlington Heights, IL, USA) following the manufacturer’s instructions.

Statistics

Data were calculated as mean ± standard deviation (SD) and analyzed by GraphPad Prism 7.0 software. One-way analysis of variance (ANOVA) was conducted with the Newman-Keuls method to determine the significance of the differences between the experimental conditions. All experiments were repeated at least three times. Differences in means were considered statistically significant when $P < 0.05$ (*), $P < 0.01$ (**), and $P < 0.001$ (***).

Results

Effects of Ssd and SP600125 on migration of U-2OS cells

To analyze the effect of Ssd and SP600125 on cell migration, we incubated the cells with drugs individually or in combination for 36 hours and performed scratch wound assays. We observed a dose-dependent increase in the inhibition of the cell migration rate by Ssd when compared to that of untreated control cells, i.e., 6.18% inhibition when Ssd was at 5 μM concentration, 11.95% when Ssd was at 10 μM concentration, and 44.64% when the Ssd concentration was 20 μM. SP600125, at 20 μM concentration, inhibited cell migration by 11.50% compared to that of the control group (Figure 1A and 1B).

Figure 1. Effects of Ssd and SP600125 alone or in combination on U-2OS cell migration. A. Cell migration was determined by wound healing assays, as described in the Methods section. The images (36 hours) are magnified by 100 ×. B. Quantification of the migration distance (cm) of SK-ES-1 cells by ImageJ software. Values are presented as the mean ± standard deviation; ***$P < 0.001$, one-way ANOVA.
Saikosaponin D and SP600125 inhibits malignant properties of U2 cells

Effect of Ssd and SP600125 on proliferation of U-2OS cells

The effect of the drugs on cell proliferation was determined by a CCK-8 assay. We observed significant inhibition in the cell proliferation rates of U-2OS cells when challenged with drugs. Cell proliferation in SP600125 (20 μM) treated cells was reduced to 86.73%, while that of cells treated with 10 μM and 20 μM Ssd was reduced to 72.22% and 50.15%, respectively, compared to the control. Combination treatment for 24 hours with 10 μM Ssd + 20 μM SP600125 reduced cell proliferation to 60.01%, and to 42.51% when cells were treated with 20 μM Ssd + 20 μM SP600125 (Figure 2A). Greater inhibition of cell proliferation was observed when drugs were used in combination rather than when used individually, implying a synergistic effect of the drugs (Figure 2B). The data also suggested that Ssd alone is more effective in inhibiting proliferation in U-2OS cells than SP600125 alone.

Ssd and SP600125 cause morphological changes in U-2OS cells

Morphological changes in U-2OS cells were measured after 24 hours of challenge with Ssd and SP600125 (0 μM Ssd/2 μl DMSO; 20 μM Ssd; 20 μM SP600125; 20 μM Ssd + 20 μM SP600125). U-2OS cells in the 20 μM Ssd group and the 20 μM Ssd + 20 μM SP600125 group were found to have concentrated and broken nuclei when stained with Hoechst 33258, which is a typical morphological feature of apoptotic cells (Figure 3A). The arrows in this figure indicate nuclear changes observed in cells. The increase in the broken nuclei morphology was most dramatic in the combination group, followed by the Ssd group, while the effect of 20 μM SP600125 on cell morphology was not as obvious.

These results were further confirmed by flow cytometry data and cell invasion experiments.

Effect of Ssd and SP600125 on apoptosis

Apoptosis was measured by flow cytometry with Annexin V-FITC/PI double labeling. The apoptotic rate (sum of early and late apoptosis) in the control group was 3.54 ± 0.44%. The apoptotic rate of cells after 24 hours of treatment with 20 μM Ssd, 20 μM SP600125, and 20 μM Ssd + 20 μM SP600125 increased to 19.24 ± 0.53, 4.81 ± 0.25, and 22.54 ± 1.41, respectively (Figure 3B).

Effects of Ssd and SP600125 on the invasion of U-2OS cells

Fewer cells were observed to infiltrate the Matrigel-coated Transwell chamber membrane after 24 hours of treatment with either 20 μM
Saikosaponin D and SP600125 inhibits malignant properties of U2 cells

Ssd or 20 μM Ssd + 20 μM SP600125, when compared to the DMSO control. The invasion corresponding to 20 μM SP600125 treatment was not statistically significant compared to that of the control group (Figure 4A and 4B).

Western blot

The expression levels of apoptosis-related proteins Bcl-2, Bax, Cytochrome C, caspase-3, caspase-8, caspase-9, PARP, and Mcl-1 were detected by western blotting. The results showed that compared to those in the control group, treatment with Ssd alone and Ssd + SP600125 caused a significant increase in Bax expression and Cytochrome C release, while Bcl-2 and Mcl-1 expression decreased (P < 0.05) (Figures 5-7). Furthermore, the expression levels of procaspase-3, -9, and -8 were downregulated, while that of cleaved caspase-3 was significantly increased (P < 0.05) (Figure 5). PARP (a key substrate for activated caspase-3) cleavage was significantly increased with drug incubation (Figure 7). Simultaneously, it was observed that the protein level of cleaved PARP in the combination group was significantly greater than that in the Ssd-alone group. The changes in Cytochrome C, cleaved caspase-3, and cleaved PARP in the SP600125 group were not statistically significant (P > 0.05).

Ssd and SP600125 reduced the expression levels of p-Akt and p-ERK. We found that the Ssd group and the Ssd + SP600125 group showed significantly reduced expression levels...
Saikosaponin D and SP600125 inhibits malignant properties of U2 cells

Discussion

In this study, we identified the anti-cancer effects and related mechanisms of Ssd and SP600125 treatment of human U-2OS cells in vitro. Wound healing assays showed that Ssd and SP600125, either individually or in combination, inhibited the migration of U-2OS cells. Results of the CCK-8 activity assay showed that the combined use of Ssd and SP600125 inhibited the proliferation of U-2OS more significantly than the two drugs alone, while Ssd had a more pronounced inhibitory effect on U-2OS cells compared to SP600125. Furthermore, Hoechst 33258 staining, Annexin V-FITC/PI staining analysis, and Transwell chamber assay further showed that Ssd alone induced apoptosis of U-2OS cells, while Ssd combined with SP600125 strongly induced the apoptosis of U-2OS cells.

Apoptosis is triggered by two distinct signals: the mitochondrial pathway and the death receptor pathway, regulated by caspase-9 and caspase-8, respectively [10, 11]. The ratio of the pro-apoptotic protein Bax to the anti-apoptotic protein Bcl-2 in the mitochondrial pathway is critical for determining the release of many apoptotic proteins (e.g., Cytochrome C) in the mitochondrial membrane space. Cytochrome C activates caspase-9 and -3. The Fas/TNF receptor of the death receptor pathway located in the cell membrane is activated by apoptosis, activating caspase-8 and -3 [12-14]. Subsequently, activated caspase-3 causes cleavage or degradation of some key cellular substrates including PARP, resulting in cell morphological changes, DNA double-strand breaks, and apoptotic cell characteristics [15, 16]. Mcl-1, an anti-apoptotic protein of the Bcl-2 family, inhibits Cytochrome C release by forming heterodimers and/or neutralizing the Bcl-2 family of proapoptotic proteins (Bim, Bak), leading to the inhibition of apoptosis. Studies have shown that downregulation of Mcl-1 protein expression can promote tumor cell apoptosis, suggesting that Mcl-1 is a potential therapeutic target for a variety of human tumors [17]. To identify the signaling pathway involved in apoptosis induced by Ssd and SP600125 alone or in combination, the levels of Bcl-2 family proteins, caspase-3, -8, and -9, and PARP were evaluated in U-2OS cells. Our data suggested that Ssd and SP600125 alone or in combina-
Saikosaponin D and SP600125 inhibits malignant properties of U2 cells


Figure 5. Effect of Ssd and SP600125 alone or in combination on the protein expression levels of procaspase-3, cleaved caspase-3, Cytochrome C, caspase-9, and caspase-8 in human U-2OS cells. A. The expression levels of procaspase-3, cleaved caspase-3, Cytochrome C, caspase-9, and caspase-8 determined by western blot analysis. GAPDH was used as the internal loading control. B-F. Quantification of Cytochrome C, procaspase-9, and cleaved caspase-8 using ImageJ software. Values are presented as the mean ± standard deviation; ***P < 0.001, one-way ANOVA.

Saikosaponin D and SP600125 inhibit malignant properties of U2 cells.

Inhibition of Akt and ERK contributes to anti-apoptotic effects and cell growth, activation of Akt or ERK plays an important role in the pathological process of cancer [18, 19]. As a proto-oncogene, the serine/threonine kinase Akt is a key component of the PI3K/Akt pathway. Activation of Akt can inhibit the mitochondrial pathway by phosphorylating the Ser196 site of the caspase-9 precursor, subsequently stopping apoptosis [20]. The results of this study indicated that Ssd, alone or in combination with SP600125, can cause a decrease in the expression of p-Akt, whereas SP600125 alone has no effect on the expression of p-Akt, suggesting that PI3K/Akt may be involved in Ssd-induced cell growth inhibition and apoptosis. ERK (extracellular signal regulated protein kinase) is an important member of the mitogen-activated protein kinase (MAPK) family and is pivotal in the cell signal transduction network.
Saikosaponin D and SP600125 inhibits malignant properties of U2 cells

Figure 6. Effect of Ssd and SP600125 alone or in combination on the protein expression levels of Bcl-2 and Bax in human U-2OS cells. A. The expression levels of Bcl-2 and Bax determined by western blot analysis. GAPDH was used as the internal loading control. B, C. Quantification of Bcl-2 and Bax using ImageJ software. Values are presented as the mean ± standard deviation; ***P < 0.001, one-way ANOVA.

Figure 7. Effect of Ssd and SP600125 alone or in combination on the protein expression levels of PARP, cleaved PARP, and Mcl-1 in human U-2OS cells. A. The expression levels of PARP, cleaved PARP, and Mcl-1 determined by western blot analysis. GAPDH was used as the internal loading control. B-D. Quantification of PARP, cleaved PARP, and Mcl-1 using ImageJ software. Values are presented as the mean ± standard deviation; ***P < 0.001, one-way ANOVA.
Karimian et al. [21] demonstrated that angiotensin II can attenuate bile acid salt-induced apoptosis by enhancing the phosphorylation activity of ERK1/2. This indicates that activation of the ERK1/2 signal transduction pathway inhibits apoptosis in the corresponding cells. Our results showed that Ssd and SP600125 alone or in combination can cause ERK1/2 protein phosphorylation inhibition, while the rate of apoptosis increases. It is suggested that the inhibition of the ERK1/2 signal transduction pathway may be involved in the cell growth inhibition and apoptosis induced by the two drugs.

In conclusion, the current data show that Ssd alone or in combination with SP600125 can induce apoptosis through the mitochondrial apoptotic pathway and the death receptor pathway, while the inhibition of U-2OS cell viability by SP600125 alone is not obvious. The combination of the two drugs has a synergistic anti-
tumor effect on U-2OS cells, and its inhibitory effect on U-2OS is better than that of Ssd alone. Taken together, these findings provide in vitro evidence to support Ssd alone and in combination with SP600125 as effective candidates for chemoprevention and/or treatment of osteosarcoma.

Further research is needed to elucidate whether the Akt pathway and the ERK pathway are involved in the mitochondrial pathway and the cell death receptor pathway. In addition, the in vivo effects of Ssd alone or in combination with SP600125 on U-2OS xenograft tumors in nude mice are currently being investigated.

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Disclosure of conflict of interest

None.

Address correspondence to: Bin Zhang and Min Dai, Department of Orthopedics, Artificial Joints Engineering and Technology Research Center of Jiangxi Province, The First Affiliated Hospital of Nanchang University, 17 Yongwai Street, Nanchang 330006, Jiangxi, P. R. China. E-mail: 13687028270@163.com (BZ); w6812039@126.com (MD)

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Saikosaponin D and SP600125 inhibits malignant properties of U2 cells

