Original Article

Selaginellin B induces apoptosis and autophagy in pancreatic cancer cells via the JAK2/STAT3 signaling pathway

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Abstract: Selaginella tamariscina (ST), a well-known traditional medicinal plant, has been used to treat various cancers, including pancreatic cancer. However, the underlying mechanism by which Selaginellin B, a natural pigment isolated and purified from ST, protects against pancreatic cells has yet to be fully elucidated. In the present study, the biological functions of Selaginellin B were investigated using apoptosis, migration and colony formation assays in ASPC-1 and PANC-1 cells. In addition, apoptosis-associated proteins were detected by Western blotting. Our results demonstrated that Selaginellin B induced apoptosis, as evidenced by the increased cleaved caspase-3 level and Bax/Bcl-2 ratio. Moreover, Selaginellin B led to a marked up-regulation of the ratio of LC3-II/LC3-I in ASPC-1 and PANC-1 cells, respectively. Furthermore, reverse pharmacophore screening, molecular docking and molecular dynamics simulation studies revealed that Janus kinase 2 (JAK2) may be a potential target for Selaginellin B. In summary, the results of the present research have demonstrated that Selaginellin B is an effective anticancer agent against PANC-1 and ASPC-1 cells, and the compound holds great promise for the treatment of pancreatic cancer.

Keywords: Selaginellin B, reverse pharmacophore screening, pancreatic cancer cell, apoptosis

Introduction

Pancreatic cancer (PC), a fatal solid malignancy with poor prognosis, exerts a significant public health burden globally. It is the fourth leading cause of mortality among all cancer cases in the United States [1-3], and is the sixth primary reason of cancer-associated mortality, with a 5-year survival rate of 1-3% in China [4, 5]. Currently, chemotherapy is of utmost importance therapy for patients with metastatic PC. Compared with gemcitabine, certain chemotherapeutic drugs, such as 5-fluorouracil, leucovorin and oxaliplatin, have achieved survival benefits for PC patients. However, serious adverse side-effects and the problem of multi-drug resistance limit their clinical applicability [6, 7]. Therefore, developing novel drugs with high efficiency and minimal adverse effects are urgently needed.

Selaginella tamariscina (Beauv.) (ST), one of the traditional Chinese herbal medicines, has been used in the treatment of cancer, hepatitis, cardiovascular diseases, and trauma [8, 9]. Selaginellin has a unique structure owing to the presence of a pigment-like scaffold [10]. In many pharmacological studies, Selaginellin and its 15 derivatives (selaginellin A-N) have shown strong biological activity, including their inhibitory effects on the proliferation of HeLa cells, as well as their protective effects against cytotoxicity in differentiated PC12 cells [10, 11]. However, the anti-PC effects of ST on cellular proliferation and the underlying mechanism(s) remain unclear.

In recent years, target fishing has emerged as a very useful technique in the campaign for the discovery of small molecular compounds with biological activities. The underlying idea of this
technique is to integrate biological information with chemical databases and then predict the biological activity of compounds through their structures. It is anticipated that computational target fishing technologies will have a profound impact on drug development and these have made target recognition more accurate and efficient [12, 13]. To date, new computational target identification methods are springing up vigorously, including reverse pharmacophore screening, molecular docking studies, and molecular dynamics (MD) simulation [13]. In the current study, these methodologies have been utilized in an attempt to identify the potential protein targets of the isolated compounds in order to further delineate the anticancer effects of selaginellin B.

Janus kinases (JAKs; namely, JAK1, JAK2, JAK3 and Tyk2) play a key role in modulating multiple signaling pathways that dominate cellular survival, differentiation, proliferation, immigration and apoptosis. JAKs/STAT signaling has been demonstrated to play a significant role in oncogenesis and development of PC [14, 15]. The mRNA levels of JAK2 and STAT3 were shown to be overexpressed in clinical PC samples. In addition, monitoring the protein levels of JAK2 and STAT3 was reported to be of clinical importance in PC tissues [15, 16]. Taken together, suppression of the JAK2/STAT3 signaling pathway has been shown to be a promising therapeutic target in PC prevention and treatment.

Therefore, the aim of this study was to investigate the anti-tumor activities of Selaginellin B on human PC cells in vitro in order to elucidate the potential mechanisms. The findings may provide novel insights into the development of potential therapeutic candidates for preventing PC.

Material and methods

Materials

Standard commercial solvents and reagents were directly used without further purifications. Silica gel was purchased from Qingdao Haiyang Chemical Co. Ltd, ESI-MS was performed on a LC-Q-TOF/MS system, $^1$H-NMR spectra were performed on Bruker DRX-400 with TMS as a reference. The primary antibodies against JAK2, phosphorylated (p)-JAK2, STAT3, p-STAT3, cleaved caspase-3, Bax, Bcl-2 and β-actin were purchased from Proteintech Group, Inc. (Chicago, IL, USA), whereas all the secondary antibodies were obtained from Abbkine Scientific Co., Ltd. (Redlands, California, USA). The Apoptosis and Cell Cycle Analysis Kit were obtained from Beyotime Institute of Biotechnology (Haimen, Jiangsu, China). All other chemicals were purchased from Sigma Chemical Co. (now a brand of Merck KGaA). Normal pancreatic ductal epithelial cells (the HPDE6-C7 cell line), and the human PC cell lines (ASPC-1 and PANC-1), were obtained from the Shanghai Institute of Biochemistry and Cell Biology (Shanghai, China).

Separation and identification of Selaginellin B

Dried powder of Selaginella tamariscina (1 kg) was reflux extracted with 95% EtOH as solvent (volume ratio of 1:5). The EtOH extract was concentrated and partitioned with petroleum ether and ethyl acetate. The ethyl acetate fraction (16.1 g) was separated by a combination of chromatographies such as silica gel column chromatography, preparative thin-layer chromatography, and preparative highperformance liquid chromatography to yield 11 compounds including Selaginellin B (6.7 mg).

Selaginellin B, red powder; ESI-MS m/z 495.25 [M + H$^+$]; $^1$HMR (400 MHz, acetone-d6): δ 6.52 (4H, d, J = 9.2 Hz), 7.13 (4H, d, J = 9.2 Hz), 7.45 (1H, d, J = 8.0 Hz), 7.23 (1H, d, J = 8.0 Hz), 6.85 (2H, d, J = 8.4 Hz), 6.63 (2H, d, J = 8.4 Hz), 7.09 (2H, d, J = 8.6 Hz), 6.73 (2H, d, J = 8.6 Hz), 2.61 (3H, s). This is consistent with the characteristics of Selaginellin B [10]. Selaginellin B was dissolved with 0.1% dimethylsulfoxide and stored at 20°C for in vitro experiments.

Cell culture

The ASPC-1, HPDE6-C7, and PANC-1 cells were cultured in high-glucose DMEM supplemented with 10% fetal bovine serum (FBS), 1% penicillin and streptomycin mixture, and maintained in an atmosphere of 5% CO$_2$ at 37°C under conditions of saturated humidity.

Cell viability assay

The human PC cells were inoculated into 96 well plates at a density of 6 × 10$^3$ cells/well and incubated overnight to allow adherence to
occur. They were subsequently treated with Selaginellin B at different concentrations (1, 5, 10 and 20 µM). Cell growth was detected at 12 h, 24 h and 48 h respectively. Each well was subsequently treated with 15 µl MTT solution (final concentration: 5 mg/ml). After incubated the reaction at 37°C for 4 h, the formazan crystals were dissolved in DMSO, then add 20 µl DMSO to each well to dissolve formazan crystals and measure the absorbance values at 490 nm by a microplate reader (Multiskan MK3; Shaanxi Pioneer Biotech Co., Ltd., Xi’an, China). Cell viability (%) was calculated using the average absorbance of different treatment groups/average absorbance of control groups × 100%. Experiments were conducted in triplicates.

**Apoptosis assay**

Apoptosis was measured using a FITC Annexin V apoptosis detection kit. Incubation of cells with different doses of Selaginellin B for 24 h. The cells were collected cells and washed twice with cold PBS, then were resuspend in 1 × Binding Buffer at a concentration of 1 × 10⁶ cells/ml. subsequently, the cells were dyed simultaneously with FITC Annexin V and PI for 15 min at room temperature in the dark. Flow cytometry was performed with a BD FACSAria™ II flow cytometer (BD Biosciences, San Jose, CA, USA) to count 10000 cells for each experiment. Flow cytometry data were finally analyzed using FlowJo version 7.6.1 (FlowJo, LLC, USA). Each experiment was carried out in triplicate.

**In vitro migration assay**

Cell migration was observed using a scratch wound (wound-healing) assay. The cells (5 × 10⁵ cells/well) were seeded into 6-well plates, cultured in an atmosphere of 5% CO₂ at 37°C under conditions of saturated humidity overnight, and subsequently scraped a line by a 200 µl aseptic pipette tip. Cells were treated with the doses of Selaginellin B indicated above in medium without FBS. After incubation for a further 24 h, cells were washed with PBS for 3 times, and images of the cells were obtained using a microscope (Nikon, Japan). The wound healing rate was calculated using the following formula: (average wound margin in 0 h - average wound margin in 24 h)/average wound margin in 0 h. Experiments were conducted in triplicates.

**Colony formation assay**

In order to verify sensitivity of the cells to Selaginellin B, a colony formation assay in vitro was used. Briefly, cells (1,000 cells/well) plated in 6 well plates were treated with different doses of Selaginellin B (0, 5, 10 µM). After incubation at 37°C for 24 h, the cells were subsequently washed with PBS after 12 h, then replenish fresh medium containing 10% fetal bovine serum. After 14 days of incubation in an atmosphere of 5% CO₂ at 37°C, the colonies were photographed using the camera (SONYDSC-W800) after staining with 0.1% crystal violet. Clones containing > 50 cells were counted for statistical analysis. Three independent assays were performed.

**Cell cycle analysis**

According to the instructions of cell cycle analysis kit. 5 × 10⁵ cells were seeded into 6-well plates, incubated with Selaginellin B. After 24 hours of treatment, the cells were collected following centrifugation and added 1 ml of ice bath precooled 70% ethanol and fix at 4°C for 2 hours or longer. The samples were then suspended with PI staining reagent (50 µg/ml PI and 1 mg/ml RNAse in 1 ml of sodium citrate buffer) at 37°C for 30 min. Finally, Flow cytometry was used to detect red fluorescence and light scattering at 488 nm. The data were finally analyzed using FlowJo version 7.6.1 (FlowJo, LLC, USA).

**Transmission electron microscopy (TEM)**

The human PC cells were plated in 6-well plates, treated with 0 and 5 µM Selaginellin B, and cultured at 37°C for 24 h. Cells were collected and fixed overnight in 0.1 M PBS at 4°C with 2% glutaraldehyde. The samples were subsequently dehydrated, embedded, sectioned. Uranium acetate and lead citrate were double dyed. Electron micrographs were recorded using a transmission electron microscope (JEM-2000EX; JEOL Ltd., Tokyo, Japan).

**Targets predicted by PharmMapper**

PharmMapper, an open-source online platform (http://www.lilab-ecust.cn/pharmmapper) for
pharmacophore matching and potential target identification, was used for the prediction of molecular targets. The molecular file of Selaginellin B was uploaded to the PharmMapper servers for in silico screening. The number of matched potential targets was set to 300, whereas remaining settings were considered as default.

**Molecular modeling**

The PDB file of Selaginellin B were generated using the PRODRG Server [17], and the protein structures were obtained from the RCSB Protein Data Bank. The protein structures were cleaned by removing crystallographic water, and prepared by the addition of polar hydrogen atoms, followed by the addition of Gasteiger charges. AutoDock 4.2 with MGL tools 1.5.6 were applied to molecular docking analysis [18], and residual interactions at the protein-drug interface were evaluated using LigPlot [19].

**MD simulation**

The conformations of the complexes formed between Selaginellin B and the potential target protein were predicted using the AutoDock 4.2 program, and each MD simulation was performed by Gromacs 5.1.5 in the Amber99sb force field. On the basis of molecular dynamics simulation, the binding free energy of Selaginellin B with potential target proteins was calculated by molecular mechanics energy binding Poisson Boltzmann specific surface area (MM/PBSA) methods.

**Western blot analysis**

Following the separation of the sample proteins by gel electrophoresis, The proteins were subsequently transferred to PVDF membranes and the membranes were incubated in a closed buffer (5% skimmed milk) at 25°C for 2 hours. Western blots were probed with the primary antibodies (1:1,000 dilution) at 4°C overnight. Then washing with TBST, the membranes were incubated with HRP conjugated secondary antibody (1:2,000 dilution) at 37°C for 1 h. The blots were then detected using enhanced chemiluminescence reagent (Thermo Fisher Scientific, Inc.), and photographs were captured using the Bio Spectrum Gel Imaging system (UVP LLC, Upland, CA, USA).

**Statistical analysis**

Statistical differences between groups were assessed using a One-way analysis of variance (ANOVA). Results are expressed as the mean ± SD. Two groups were compared with a two-tailed Student’s t-test using Graph Pad Prism 5 (Graph Pad Software, Inc., San Diego, CA, USA). Bonferroni’s test was used to correct multiple comparisons, and $P$ values <0.05 were considered statistically significant.

**Results**

**Structural elucidation**

Selaginellin B was obtained as a red powder, with the molecular formula $C_{34}H_{24}O_{4}$, deduced from ESI-MS on the basis of the quasi-molecular ion peak at $m/z$ 495.25 [M + H]. The $^1$H NMR data of Selaginellin B were consistent with previous studies (Figures 1A and S1) [10].

**Effect of Selaginellin B on cell viability and morphology of PC cells**

To identify the cytotoxic effect of Selaginellin B in HPDE6-C7 and ASPC-1 and PANC-1 cells, the cells were treated with different concentrations of Selaginellin B for subsequent analysis using MTT assay (Figure 1C-E). The concentration of the drug that led to 50% inhibition (i.e., the IC$_{50}$ value) following 24 h of Selaginellin B treatment was determined to be 6.4 μM in the ASPC-1 cells, and 8.8 μM for the PANC-1 cells. In addition, the results demonstrated that Selaginellin B suspended the cell viability of the ASPC-1 and PANC-1 cell lines in both concentration- and time-dependent manner. However, Selaginellin B showed less toxicity towards HPDE6-C7 cells, suggesting that Selaginellin B may be a promising anti-PC agent. Furthermore, treatment with Selaginellin B led to marked cellular morphological changes, including rounding and blebbing of the cells, and a reduction in cell-to-cell interactions, as shown in Figure 1B.

**Selaginellin B induces apoptosis in the pancreatic cancer cells**

To examine whether the antitumor activity of Selaginellin B was associated with apoptosis in PC, Annexin V-FITC and PI double staining was performed using flow cytometry analysis. Marked increases in the apoptotic rates were
observed, from 6.69% (in the control group) to 12.01, 22.90, and 41.70% in the ASPC-1 cells, and from 2.69% (control group) to 8.46, 17.07, and 24.05% in the PANC-1 cells, upon treatment for 24 h with 1, 5 and 10 µM Selaginellin B, respectively (Figure 2A). The levels of apoptosis-associated proteins were subsequently investigated by Western blot analysis. The ratio of Bax/Bcl-2, which regulates apoptosis by regulating mitochondrial membrane permeability [16], was increased by Selaginellin B. The levels of procaspase-9 and procaspase-3 were shown to be inhibited, whereas that of cleaved caspase-3 was increased, by Selaginellin B (Figure 2B). These results suggested that Selaginellin B could induce apoptosis in ASPC-1 and PANC-1 cells in a concentration-dependent manner.

Effect of Selaginellin B on cell cycle distribution of ASPC-1 and PANC-1 cells

To explore the cancer cell growth inhibitory mechanisms of Selaginellin B, subsequently, the effect of Selaginellin B on cell cycle arrest in PC cells was examined. The results showed that Selaginellin B treatment caused an accumulation of the two PC cell lines in the G1-phase. As shown in Figure 3, the percentages of ASPC-1 and PANC-1 cells in the G1-phase were increased from 42.88% in the control treatment group to 55.91% in the 10 µM Selaginellin B group for the ASPC-1 cells, and from 53.41% in the control treatment group to 69.67% in the 10 µM Selaginellin B group for the PANC-1 cells. These findings suggested that Selaginellin B could induce G1-phase arrest of the PC cells.

Suppression of the migration and colony formation of PC cells by Selaginellin B

As shown in Figure 4A after 24 h of incubation, for the control groups of the migrating cells (ASPC-1 and PANC-1 cell lines), the wound between cell layers formed after the scratch was made was almost entirely occupied by cells, whereas for the groups that were treated with Selaginellin B, the cells were prevent-
Anticancer effect of Selaginellin B on PC in vitro

Figure 2. Apoptosis induction of Selaginellin B in the pancreatic cancer cells. A. Flow cytometric analysis of the apoptosis induction of Selaginellin B in the ASPC-1 and PANC-1 cells. After treatment with Selaginellin B at concentrations of 1, 5 and 10 μM for 24 h, the cells were stained with annexin V-FITC and PI, and subsequently analyzed by flow cytometry. The dot plots have been obtained from 1 out of 3 independent experiments, performed in the same experimental conditions, which gave similar results. The values are expressed as the mean ± SD of 3 independent experiments. *P<0.05; **P<0.01 vs. the control group. B. Western blot analysis was performed to determine the apoptosis pathway induced by Selaginellin B in the ASPC-1 and PANC-1 cells. Cells were treated with different concentrations of Selaginellin B (1, 5 and 10 μM) for 24 h.
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Figure 3. Analysis of the cell cycle distribution of Selaginellin B-treated ASPC-1 and PANC-1 cells. Cells were treated with different concentrations of Selaginellin B (1, 5, and 10 μM) for 24 h, and cell cycle distribution was subsequently analyzed by flow cytometry.
Anticancer effect of Selaginellin B on PC in vitro

Figure 4. Selaginellin B suppressed cell migration and colony formation. A. Cell migration was analyzed by wound-healing assay. ASPC-1 and PANC-1 cells were seeded in 6-well plates, and grown until they were fully confluent. The cell migration rate was quantified. Scale bar = 100 μm. B. The ability of the ASPC-1 and PANC-1 cells to induce colony formation was also analyzed (Scale bar = 5 mm), and the colony formation rate was calculated. (All data are presented as the mean ± SD, n = 3, **P<0.01; ***P<0.001 vs. the control group).
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Selaginellin B induces autophagy in PC cells

The micro-morphological changes of Selaginellin B-treated PC cells were observed by TEM, which has been demonstrated to be the most convincing method standardly employed to verify autophagy [20-22]. An intact cell and nuclear membrane architecture was observed, and the nucleus, homogeneous cytoplasm remained normal as far as the ultra-structural morphology of the untreated cells was concerned, whereas treatment with 5 µM Selaginellin B resulted in the presence of numerous vacuoles in the cytoplasm of the autophagosomes in the PC cells (Figure 5A). Microtubule-associated protein 1A/1B-light chain 3 (LC3), the specific marker of autophagy, was also detected by Western blot analysis (Figure 5B). Compared with the control group, Selaginellin B treatment increased the ratio of LC3-II/LC3-I (i.e., of the lipid-modified form to the cytosolic form). These data showed that Selaginellin B induced autophagy in both ASPC-1 and PANC-1 cells in a concentration-dependent manner. Furthermore, to provide an insight into the relationship between apoptosis and autophagy induced by Selaginellin B, the level of cleaved caspase-3 was evaluated following pre-treatment with chloroquine, an autophagy late-stage inhibitor. These results showed that pre-treatment with 10 µM chloroquine led to a marked increase in the expression of cleaved caspase-3, as compared with the group that was only treated with Selaginellin B (5 µM) (Figure 6). These data suggested that Selaginellin B-induced autophagy prevented cells from undergoing Selaginellin B-induced cell death, further inhibited apoptosis triggered by Selaginellin B in the ASPC-1 and PANC-1 cells.

Potential protein targets for Selaginellin B

First, potential protein target for Selaginellin B identified by virtual screening procedures with PharmMapper and the top 200 hits were sought after. Additionally, literature sources and gene database systems were consulted to decide whether the potential targets identified were associated with PC. Among the protein targets, 68 revealed a positive response. Moreover, MD simulation analysis was performed to determine the potential binding modes between Selaginellin B and the 68 protein targets. The results of the top 10 potential protein targets are as follows:
Anticancer effect of Selaginellin B on PC in vitro

Figure 6. Effect of autophagy on the apoptosis of Selaginellin B-treated ASPC-1 and PANC-1 cells. Cells were pretreated with chloroquine (10 µM) for 2 h, and then treated with Selaginellin B (5 µM) for 24 h. A. Cell apoptosis was measured by flow cytometry. And the percentage of apoptotic cells was quantified. The dot plots have been obtained from 1 out of 3 independent experiments, performed in the same experimental conditions. Histogram data are presented as mean ± SD, n = 3. *P<0.05; **P<0.01 vs. the control group. B. Western blot analysis of LC3 and cleaved caspase-3 protein expression. Cells were pretreated with chloroquine (10 µM) for 2 h, and then treated with Selaginellin B (5 µM) for 24 h.
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Selaginellin B inhibits JAK2 activity by targeting the ATP-binding site

STAT3 is activated through cytokines and receptor tyrosine kinases, especially JAK2. One of the integral signaling mechanisms for pancreatic carcinogenesis has been shown to involve JAK2 [14, 23]. Therefore, the effect of Selaginellin B on the protein level of JAK2 was investigated by Western blot. As shown in Figure 7B, Selaginellin B significantly suppressed the protein expression levels of both p-JAK2 and p-STAT3 in a concentration-dependent manner. In order to further confirm the role of Selaginellin B in regulating the JAK2/STAT3 signaling pathway in PC cells, ASPC-1 and PANC-1 cells were treated with Selaginellin B (5 μM) following pre-treatment with the JAK2 inhibitor, Fedratinib (1.5 μM) for 4 h. Figure 7C showed the levels of JAK2/STAT3 were significantly decreased, whereas the levels of an apoptosis-associated protein (cleaved caspase-3) were markedly increased. Furthermore, the results obtained from the docking simulation revealed what was the top-ranking docked conformation, with low binding affinity (-9.78 kcal/mol), and Selaginellin B was embedded in the active site of JAK2, fitting well within the cavity in three-dimensional space and interacting with key amino acid residues in the binding domain (Figure 7D and Movie S1). The fundamental interactions (highlighting the hydrogen bonds and hydrophobic interactions) are shown in Figure 7E, prepared using LigPlot. Specifically, in the ATP-binding site of the JAK2 kinase domain, Selaginellin B interacts with the active-site amino-acid residues (Asp939, Lys857, Asn859, and Phe860), forming four hydrogen bonds. Furthermore, Selaginellin B maintains hydrophobic interactions with the catalytic residue Asp976 and its surrounding residues, Gly861, Gly858, Asp994, Asn981, Gly993, Leu983, Gly856, Gly935, Arg980, Ser936, and Lys882, in the active site. Collectively, these results suggest that Selaginellin B inhibits JAK2 activity by targeting the ATP-binding site.

Discussion

PC is a highly lethal malignancy, and the treatment outcomes following chemotherapy at present are rather poor [24, 25]. A previous study indicated that ST exerts its anti-cancer effects against a panel of human cancers, such as breast cancer, leukemia and cervical cancer [26]. Similarly, the present study demonstrated that Selaginellin B markedly inhibits cell viability and induces cell apoptosis in ASPC-1 and PANC-1 cells. Interestingly, Selaginellin B barely exerted activities on HPDE6-C7 normal pancreatic cells, suggesting that Selaginellin B could be invoked as a protective agent in therapies against PC.

In multicellular organisms, there exists a delicate balance between the cell-generating ef-
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A

![Bar graph showing the PBBA Binding Free Energy (kJ/mol) for various proteins.]

B

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C

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D

![3D molecular structure showing interactions and bonds.]

E

![Close-up of molecular interactions and bonds with labels.]

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Effects of mitosis and apoptosis-induced cell death [27-29]. Currently, both of apoptosis and cell cycle arrest are important processes that are exploited by anticancer drugs [30, 31]. Consequently, flow cytometric analysis was performed to assess whether Selaginellin B induced cell death of the PC cells was associated with apoptosis. In this study, Selaginellin B markedly induced apoptosis in a concentration-dependent manner in the PC cells. According to the existing evidence, the receptor-mediated (extrinsic) and mitochondria-associated (intrinsic) pathways are the two major apoptotic cell death pathways [32]. ST has been shown to possess numerous pharmacological benefits, including the mediation of anti-bacterial, anti-inflammatory and anti-cancer effects [33-35]. The respective mechanisms of these pharmacological effects have been shown to be associated with the release of cytochrome c from the mitochondria, characterized by increases in the Bax/Bcl-2 ratio, caspase-9 and caspase-3 activity [36-38]. The action of Selaginellin B has been linked with intrinsic apoptosis, which is mediated via targeting the mitochondria.

The cell cycle, featuring its various components and stages, and cell growth and division, forms a complex biological process [39, 40]. In the present study, Selaginellin B induced anti-proliferative effects on pancreatic cells by restricting cell cycle progression, which was demonstrated through blocking G0/G1 phase in ASPC-1 and PANC-1 cells.

Several natural products have been demonstrated to exert anticancer activity, mainly via autophagy-dependent mechanisms [41-43]. Autophagy, as an evolutionarily conserved lysosomal degradation process, has an important role in regulating pro-survival and pro-death signaling pathways in numerous diseases, especially cancer. In the present study, as revealed by TEM, the autophagosomes of the ASPC-1 and PANC-1 cells upon treatment with Selaginellin B exhibited the typical appearance of containing cytoplasmic organelles and an engulfed bulk cytoplasm. Furthermore, a marked conversion of LC3I to LC3II was also detected [44, 45]. Therefore, in order to determine the association between autophagy and apoptosis induced by Selaginellin B, the extent of apoptosis in the cells, and the level of cleaved caspase-3 following pre-treatment with chloroquine, were evaluated. The findings revealed that apoptosis triggered by Selaginellin B is significantly increased following the blockade of autophagy, suggesting that autophagy induced by Selaginellin B may serve as an inhibitor of apoptosis in ASPC-1 and PANC-1 cells.

In recent years, target fishing technologies fulfill an important role in investigating the mechanism of bioactive small molecules [46, 47]. In this study, the potential protein targets of Selaginellin B were detected via reverse pharmacophore screening, molecular docking study and MD simulation. The results obtained revealed that JAK2 was the best potential protein target with the lowest PBSA binding free energy (-182.91 kcal/mol). Furthermore, the
binding model revealed that electrostatic interactions and hydrogen bonds may contribute to the binding of Selaginellin B in the ATPase catalytic site of JAK2. Therefore, Selaginellin B may possibly act as a competitive inhibitor via JAK2, and may serve as a potential antitumour drug in the future.

Several types of tumor pathogenesis have been reported due to the aberrant dysregulation of JAK/STAT signaling. In addition, JAK2/STAT3 signaling is greatly activated in PC tissues, and serves an important role in regulating cell proliferation, apoptosis, and oncogenesis in cancer cells [48-51]. However, the molecular mechanisms associated with JAK2/STAT3 signaling in apoptosis have not yet been fully explained. The results of the present research revealed that Selaginellin B was able to suppress the expression of both p-JAK2 and p-STAT3 in ASPC-1 and PANC-1 cells in a dose-dependent manner. Compared with treatment with Selaginellin B or Fedratinib alone, treatment with both Selaginellin B and Fedratinib led to a marked increase in the levels of cleaved caspase-3, and a significant decrease in the levels of p-JAK2 and p-STAT3, indicating that the anti-proliferative effects of Selaginellin B are largely dependent on JAK2/STAT3 pathway inactivation.

In conclusion, the present study has demonstrated, for the first time, that Selaginellin B significantly decreased the cell viability of ASPC-1 and PANC-1 cells with minimal cytotoxicity in normal HPDE6-C7 cells, suggesting that Selaginellin B can selectively kill PC cells. Our results also revealed that Selaginellin B exerted its anti-tumor activity through cell cycle arrest and autophagy, which results in apoptosis via the JAK2/STAT3 signaling pathway (Figure 8). Therefore, Selaginellin B might provide a novel therapeutic option for the treatment of PC.

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Disclosure of conflict of interest

None.

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**Figure S1.** The purity of Selaginellin B was confirmed by ESI-MS, $^1$H-NMR and HPLC analyses. A. ESI-MS results, showing the molecular mass of Selaginellin B. B. Proton NMR ($^1$H-NMR) analysis, revealing the exact integration and positions of the protons pertaining to Selaginellin B. C. HPLC profile of Selaginellin B, revealing the purity to be 98.7%.

**Movie S1.** Movie of 20 ns of MD simulation of Selaginellin B complexed in the pocket of JAK2. JAK2 is shown in cyan, whereas Selaginellin B is shown in the orange sphere model. MD, molecular dynamics; JAK2, Janus kinase 2.