Icariside I reduces breast cancer proliferation, apoptosis, invasion, and metastasis probably through inhibiting IL-6/STAT3 signaling pathway

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Abstract

Objectives: Breast cancer is a common malignancy in women. More than 90% of breast cancer deaths are caused by metastasis. Epimedi Folium (EF) is a commonly used herb with anti-tumor benefits, but its underlying mechanisms and active components for breast cancer prevention are little understood. This study assessed the therapeutic role of Icariside I (ICS I) in Epimedium flavonoids (EF) on lung metastasis of breast cancer, including the underlying mechanism.

Methods: Western blot, RT-qPCR, wound healing assay, colony formation assay, and flow cytometry were used to investigate the inhibition of breast cancer cells growth and migration by EF and ICS I through disrupting the IL-6/STAT3 pathway. Combined with 4T1 breast cancer model in mice. Western blot, RT-qPCR, Hematoxylin and Eosin staining, immunohistochemistry were used to evaluate the therapeutic role of ICS I in proliferation, apoptosis, invasion, and metastasis of breast cancer.

Key findings: EF can inhibit STAT3 phosphorylation and reduce the colony formation and migration of breast cancer cells. Detecting the active ingredients in EF, we found ICS I can reduce the activation of STAT3 in 4T1 breast cancer cells, impair colony formation and migration. Moreover, ICS I induced cells G1 phase arrest and modulated Cyclin D1, CDK4, bcl-2, and bax to inhibit proliferation and survival of breast cancer cells. Similarly, the in vivo studies demonstrated that ICS I significantly suppressed tumor development and lung metastasis in the 4T1 mouse model. Tumor cells in vehicle group were arranged in a spoke-like pattern with obvious heterogeneity, and multinucleated tumor giant cells were seen. But, the tumor cells in the ICS I group were disorganized and necrotic lysis was seen in some areas. In ICS I-treated group, tumour cells' STAT3 phosphorylation level, IL-6, Cyclin D1, CDK4, bcl-2, and vimentin expression were downregulated, bax and cleaved caspase 3 expression were upregulated. In the lung tissue, we could find less metastasis of breast cancer cells and less lung injury in the ICS I group. Besides, the expression of metastasis-related genes MMP9 and vimentin was decreased in the lung tissue of ICS I group.

Conclusions: These findings suggest that ICS I can inhibit breast cancer proliferation, apoptosis, invasion and metastasis probably via targeting IL-6/STAT3 pathway. Therefore, ICS I has the potential to become an innovative therapeutic candidate to breast cancer prevention and treatment.

Keywords: Icariside I, breast cancer, IL-6, STAT3, cancer growth; cancer metastasis

Introduction

Breast cancer is still a serious public health concern around the world. It continues to pose a serious threat to women’s health and well-being [1]. Breast cancer treatments such as radiotherapy, chemotherapy, and hormone therapy all have unavoidable side effects [2, 3]. Despite advancements in the development of medication and mechanisms, people with breast cancer still have a poor clinical prognosis and survival rate [4]. Metastasis is often the last fatal step in breast cancer progression [5]. Breast cancer targeted medicines have advanced considerably in recent decades. Breast cancer cells’ origin, progression, apoptosis, and metastasis are all connected to the IL-6/STAT3 pathway [6].

Interleukin 6 (IL-6) is a multifunctional cytokine that impacts practically every organ system, particularly the immune system [7]. It is thought to be a key factor in cancer development and metastasis. A study found that the IL-6 system could be a potential target for tumor immunotherapy [8]. When IL-6 binds to the IL-6 receptor (IL-6R), the IL-6R undergoes a conformational change. The IL-6R/gp130 protein complex is formed when IL-6R binds securely to the extracellular end of gp130 [9, 10]. Then gp130 dimers and
activates Janus kinase 2 (JAK2), causing signal transducer and activator of transcription 3 (STAT3) to be phosphorylated [11]. STAT3 that has been phosphorylated produces a dimer and translocates to the nucleus. Overactivation of the STAT3 pathway, which is initiated by IL-6 and generally associated with a poor prognosis, has been linked to breast cancer [12]. By controlling the expression of downstream target genes (bcl-2, bax, Cyclin D1, MMP9, MMP2, and others), abnormal STAT3 signaling accelerates breast tumor progression [13]. Thus, targeting the IL-6/STAT3 pathway in breast cancer patients may be beneficial in terms of reducing tumor cell growth and enhancing antitumor immunity [14]. These results implied that medicines targeting IL-6/STAT3 may provide novel breast cancer therapeutic methods.

Recently, there has been great interest in the development of anticancer drugs using natural ingredients. Epimedi Folium, derived from the dried leaves of *Epimedium brevicornum* Maxim., *E. sagittatum* (Sieb. et Zucc.) Maxim., *E. pubescens* Maxim., and *E. koreicum* Nakai., is frequently employed as a tonic, laxative, anti-rheumatic, and anti-cancer agent in traditional Chinese and other Asian medicinal herbs [15]. In China, Epimedi Folium is considered by National Health Commission of the People’s Republic of China (http://www.nhc.gov.cn/) to be the homology medicine and food, which could be found in a variety of foods, including tea and condiments [16]. In China, Epimedi Folium is commonly used to treat breast cancer in clinic. However, the material basis and mechanism of Epimedi Folium in treating breast cancer need to be further explored. Modern pharmacological investigations have demonstrated that Epimedium flavonoids (EF) are the primary active components of Epimedi Folium. Icariside I (ICS I) is an active flavonoid, one of the principal metabolic constituents of Epimedi Folium [17]. It has some biological and pharmacological functions, including anti-osteoporosis and anticancer. Its anticancer activity was reported in melanoma. By regulating gut microbiota and host immunology, ICS I was discovered to dramatically limit the progression of B16F10 melanoma [18]. However, the specific anticancer mechanisms of ICS I remain to be clarified. STAT3 is a transcriptional activator in breast cancer, which affects the progress, proliferation, apoptosis, metastasis, and treatment resistance of breast cancer. STAT3 inhibitor alone or in combination with other clinical drugs can provide a good effect for inhibiting or reversing breast cancer. We conducted this study to consider whether ICS I is the effective ingredient of Epimedi Folium for anti-breast cancer. Considering the importance of IL-6/STAT3 pathway in the development of breast cancer, we thought about whether ICS I inhibited the growth and metastasis of breast cancer by downregulating the level of STAT3 phosphorylation.

The anti-breast cancer properties and underlying mechanisms of ICS I, one of the key active and metabolic compounds in EF, were studied in this research. By inhibiting IL-6-induced STAT3 phosphorylation, our research indicated that ICS I might lead to growth arrest and metastasis suppression, suggesting that ICS I is a promising candidate for lung metastatic breast cancer treatment in humans.

### Materials and methods

#### Experimental animals

Female BALB/c mice aged 6 weeks were acquired from Beijing SPF Biotechnology Co., Ltd (China). Before the trial began, the animals were given 1 week to adapt and had free access to food and water. At the Fifth Medical Center, Chinese PLA General Hospital, there were places for animals to live. In this work, all animals care and experimental procedures adhered to the Guide for the Care and Use of Laboratory Animals of the Fifth Medical Center, Chinese PLA General Hospital. This study was examined and approved by the Animal Ethics Committee of the Fifth Medical Center, Chinese PLA General Hospital (Beijing China). The ethical approval number was IACUC-2021-0007. The ethical approval date was 12 March 2021.

#### Chemicals and reagents

Sigma-Aldrich (Munich, Germany) was the supplier of dimethyl sulfoxide. Epimedium flavonoids were purchased from SHAAN XI HONGTAI BIOTECHNOLOGY CO., LTD. Icaritin (HY-N0014, purity 99.06%), Icaritin (HY-N0678, purity 99.27%), Sagittatoside A (HY-N0873, purity 99.72%), Sagittatoside B (HY-N0874, purity ≥ 99.0%), Epimedin A (HY-N0257, purity 99.87%), Epimedin A1 (HY-N0258, purity 99.85%), Epimedin B (HY-N0259, purity 99.90%), Epimedin C (HY-N0260, purity 99.47%), Baohuoside II (HY-N0875, purity 99.27%), and Icariside II (HY-N0011, purity 99.15%) were supplied by MedChemExpress (NJ, USA). Icariside I (56725-99-6, purity 99.47%) was purchased from Targetmol (Boston, USA). Anti-p-STAT3 (Tyr705) (1:1000, ab76315) and anti-Cyclin D1 (1:2000, ab16663) were supplied by Abcam (Cambridge, UK). Anti-STAT3 (1:2000, 4904) and anti-vimentin (1:2000, 5741) were purchased from Cell Signaling Technology (Boston, USA). Anti-beta-actin (1:2000, 4056S-1-AP), anti-caspase 3/p17/p19 (1:1000, 66470-2-lg), and anti-bax (1:2000, 50599-2-lg) were purchased by Proteintech (Chicago, USA). Anti-CDK4 (1:2000, PB9535) was obtained from Boster Biological Technology Co. Ltd (California, USA). Santa Cruz Biotechnology (Dallas, USA) was the vendor for the acquisition of anti-bcl-2 (1:250, sc7382). Human IL-6 (200-06) was obtained from PepProTech (New Jersey, USA). Cell Cycle and Apoptosis Analysis Kit (C1052) were supplied by Beyotime (Shanghai, China). CellTiter-Glo® Luminescent Cell Viability Assay (G7572) was purchased from Promega. GenStar (Beijing, China) supplied the StarRuler Color Prestained Protein Marker (10-180kDa) (M221). Certified Fetal Bovine Serum (C04001) was supplied by VivaCell (Shanghai, China).

#### Animal experiment protocol

4T1-luc cells (1 × 10^5) were suspended in phosphate-buffered saline and subcutaneously injected into the fourth breast pad of female BALB/c mice. On Day 5, following cell inoculation, mice were randomized into three groups (n = 6) and given intragastric administrations of CMC-Na, ICS I (25 mg/kg), and ICS I (50 mg/kg). The tumor volume was routinely assessed and expressed in mm^3, calculated using a standard formula (length × width^2)/2. After drug administration for 27 days, solid tumors and lungs were removed. They were stored in a 4% formalin solution. Then the samples were then stained with H&E and the target proteins were detected by immunohistochemistry.

#### Cell culture and treatment

The 4T1-luc (4T1) murine breast cancer cells were cultured in a 5% CO_2 incubator at 37°C using Roswell Park Memorial Institute (RPMI) 1640 medium (Mgacene) supplemented.
with 10% fetal bovine serum and 1% penicillin-streptomycin (Macgene).

**Cell viability test**
In 96-well plates, 4T1 cells were planted overnight at a density of 2 × 10⁴ cells/mL. Cell viability was then tested by measuring ATP levels after incubating for 12 h with various doses of ICS I (0-100 μM).

**Medicine impact detection**
4T1 at a density of 2.5 × 10⁴ cells/mL were planted into 24-well plates for 12 h. The 4T1 cells were serum-deprived for a period of 12 h using a medium that did not include any serum. The cells were then pretreated with 10, 20, and 40 μM ICS I for a period of 1 h each. Following a 12 h treatment with IL-6 at a concentration of 50 ng/mL, cell lysates were extracted and collected for western blotting and RT-qPCR analysis.

4T1 cells at 2.8 × 10⁴ cells/mL were cultured in 12-well plates overnight. Following the application of the same treatment, the cells were harvested, then stained in accordance with the instructions of the Cell Cycle and Apoptosis Analysis Kit. Flow cytometry was used to analyze the cells.

**Colony formation assay**
To summarize, about one thousand 4T1 cells were planted into a six-well plate and then kept in an incubator at 37°C for one night. The 4T1 cells were then treated with ICS I (10, 20, and 40 μM) or EF (0.5, 1, 2 mg/mL) and IL-6 (100 ng/mL) for 5 days in an incubator. At room temperature, the cells were subsequently fixed with 4% paraformaldehyde for 15 min and then stained with 1% crystal violet for 15 min. Double distilled water was used to wash the cells. Finally, the numbers of cell colonies were counted.

**Western blotting**
The cells were dissolved with 1 x RIPA sample loading buffer, separated by 10% and 12% SDS-PAGE gel electrophoresis. The samples were subsequently subjected to gel electrophoresis with 10% or 12% SDS polyacrylamide. Polyvinylidene difluoride (PVDF) membranes were used to adsorb proteins on the gel. The membrane was placed on a shaker and incubated in 5% skimmed milk at room temperature for 1 h. Following this step, they were treated with the antibodies listed below: p-STAT3 (Tyr705), STAT3, and vimentin. β-actin was used as a loading control. After incubation with horseradish peroxidase (HRP)-conjugated secondary antibody diluted in blocking solution, it was detected by ECL luminescence and developed on film.

**Real-time quantitative polymerase chain reaction**
In order to measure the level of mRNA expression in 4T1 cells, mouse tumors and lung tissues, Q-PCR analysis was utilized. Total RNA cells were extracted from 4T1 cells, mouse tumors and lung tissues by TRIZOL (Bioflux) reagent. RT Master Mix (HY-K0510A, MCE) was used to reverse transcribe RNA into cDNA. After adding SYBR Green qPCR Master Mix (HY-K0522, MCE), it was evaluated by real-time fluorescence quantitative PCR system of an Applied Biosystems ViiA6 Real-time PCR system. The qPCR reaction is 95°C for 5 min, 40 cycles at 95°C for 10 s, 60°C for 20 s, and 72°C for 20 s, then 1 cycle at 95°C for 15 s, 60°C for 1 min, and 95°C for 15 s. The sequences of PCR primers are presented in Table 1.

**Wound healing assay**
After placing wound healing 4-well inserts in 12-well plates, 4T1 cells were inoculated at a density of 4 × 10⁴/mL. After cell adhesion, inserts were removed and phosphate-buffered saline was used to remove floating dead cells. Next, serum-free RPMI 1640 medium with or without ICS I (10, 20 or 40 μM) or EF (0.5, 1, 2 mg/mL) and IL-6 (100 ng/mL) were added. The cultures were kept in an incubator at 37°C for 24 h. At 0 and 24 h, photographs were taken with an inverted microscope. ImageJ was used to analyze the pictures (http://rsb.info.nih.gov/ij/). The following method was used to count the migration of cells to the wound scratch: Percentage of migrated cells = [(A1 – A2)/A1] × 100%, where A1 is the wound area for 0 h and A2 is the wound area for 24 h.

**Statistical analysis**
Data were presented as mean ± standard error (SEM). GraphPad Prism 8.0 (GraphPad Software) was used for statistical analysis. One-way ANOVA for multiple groups was used to assess significant differences. A P-value < .05 was considered statistically significant.

**Results**
EF suppresses STAT3 phosphorylation, proliferation, and migration in 4T1 cells
In addition to its important part in both cancer and inflammation, the transcription factor STAT3 has been linked to the

<table>
<thead>
<tr>
<th>Target gene</th>
<th>Sequence (5’−3’)</th>
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<tbody>
<tr>
<td>mouse Cyclin D1</td>
<td>Forward TGACTCGCGAAGATGTCTGCG</td>
</tr>
<tr>
<td>mouse CDK4</td>
<td>Forward ATGGGCTGACCTCGATAGAA</td>
</tr>
<tr>
<td>mouse MMP2</td>
<td>Forward ACCTGAAACATTCTTTAGCTG</td>
</tr>
<tr>
<td>mouse MMP9</td>
<td>Forward GAGCCCGAAGCGGACATTG</td>
</tr>
<tr>
<td>mouse bcl-2</td>
<td>Forward GCTACCTGCTGACTTCCG</td>
</tr>
<tr>
<td>mouse bax</td>
<td>Forward AGACAGGGGCTTTTTGTGC</td>
</tr>
<tr>
<td>mouse vimentin</td>
<td>Forward CTGCTTCAGACTCGGTGGAC</td>
</tr>
<tr>
<td>mouse GAPDH</td>
<td>Forward AGGTCCGGTGAACCCAGTGGT</td>
</tr>
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Table 1. Primer sequences for Real−time quantitative polymerase chain reaction.
onset, progression, and metastasis of breast cancer [19, 20]. We first examined the effects of flavonoids prepared from Epimedi Herba on IL-6- induced expression of p-STAT3 in 4T1 by cell-Western blotting. The EF significantly inhibited IL-6-induced STAT3 phosphorylation (Fig. 1A). Next, the EF was added to IL-6-induced breast cancer cell colony formation. It was discovered that EF had a strong inhibitory effect on the development of colonies in 4T1 breast cancer cells, which were stimulated by IL-6 (Fig. 1B and C). Furthermore, EF also inhibited IL-6-induced wound healing (Fig. 1D and E). These data indicated that EF has a potentially inhibitory effect on STAT3 phosphorylation, proliferation and migration of 4T1.

ICS I inhibits IL-6/STAT3 signaling in breast cancer cells

Icarin, Icaritin, Sagittatoside A, Sagittatoside B, Epimedin A, Epimedin A1, Epimedin B, Epimedin C, Baohuoside II, ICS I, and Icariside II are the main active compounds of EF. They were investigated to test their effect on IL-6/STAT3 signaling. Icaritin, ICS I and Icariside II can reduce the phosphorylation of STAT3 induced by IL-6 (Fig. 2A). This result suggested that Icaritin, ICS I and Icariside II may be the main components in EF that inhibited the proliferation and migration of breast cancer cells. Numerous studies have demonstrated the anti-tumor efficacy of Icaritin and Icariside II [21, 22]. However, fewer anticancer investigations have been conducted on ICS I. In this work, we investigated the anti-tumor mechanism of ICS I (Fig. 2B). To determine if ICS I had a cytotoxic impact on breast cancer cells, the Cell Titer-Glo Luminescent Cell Viability Assay was conducted. In Fig. 2C, ICS I at a concentration lower than 60 μM did not impair the viability of 4T1 cells after treatment for 12 h. The level of p-STAT3 (Tyr705) decreased in 4T1 cells that had been pretreated with ICS I (10, 20 and 40 μM) for 1 h and then exposed to IL-6 (50 ng/mL) after 12 h (Fig. 2D). According to the findings, ICS I was able to inhibit IL-6/STAT3 signaling in 4T1 cells.

ICS I suppresses proliferation and migration in breast cancer cells

The above results suggested that ICS I effectively inhibited the activation of STAT3. Next, we investigated whether ICS I may alter the proliferation and migration of 4T1 cell. We then used a wound-healing test to study the impacts of ICS I on cell migration in 4T1 cells. Our data attested that treatment with 10, 20 and 40 μM of ICS I produced a concentration-dependent inhibition of IL-6-induced wound healing (Fig. 3A and B). Colony formation tests were conducted to determine the proliferative and regenerative capacity [23]. It was discovered that ICS I had a strong inhibitory effect on the development of colonies in 4T1 breast cancer cells, which were stimulated by IL-6 (Fig. 3C and D). In addition, STAT3 is an essential component in the process of controlling cell migration since it is responsible for controlling the production of genes that are involved in MMP2, MMP9, and vimentin [24]. By dissolving collagen IV and causing structural damage to the vascular basement membrane, MMP9 and MMP2 can enhance the likelihood of tumor spread by damaging peritumor tissue [25]. Vimentin also affects cancer cell motility and migration [26]. Consequently, as Fig. 3E indicated, in the presence of IL-6, the protein level of vimentin was decreased under the treatment of ICS I. A real-time quantitative polymerase chain reaction (RT-qPCR) assay showed that ICS I also reduced the mRNA level of MMP2 and MMP9 (Fig. 3F and G). Altogether, our results indicated that ICS I exhibited a potent capacity to suppress breast cancer cell migration and proliferation.

ICS I induces G1 phase cell cycle arrest and affects STAT3 downstream target genes in breast cancer cells

STAT3-mediated expression of critical target genes that govern cell proliferation is the primary mechanism by which STAT3 activation affects tumor cell growth [14, 27]. Cell proliferation is regulated by the cell cycle, which is divided into G1, S, and G2/M phases [28]. Flow cytometry on PI-stained cells also showed the effect of ICS I on IL-6- induced cell cycle progression. 4T1 cells were placed in a medium without serum for 12 h, treated with ICS I for 1 h, and then cultured for 12 h with or without IL-6 (50 ng/mL). When deprived of serum, as observed under control conditions, 4T1 cells entered a viable G1 arrest state. In the presence of IL-6, the proliferative phase of the cell cycle (S+G2/M) was increased in 4T1. In contrast, administration of ICS I completely antagonized this effect, preventing breast cancer cells from entering the S phase (Fig. 4A). 4T1 cells were treated with ICS I alone in Supplementary Fig. S1. This might mask the direct effect of ICS I on the cell cycle. In the single-administration group, the G1 phase was 43.6% in control group while 49% in the 40 μM ICS I administration group. However, with IL-6 induction, the G1 phase in the control group was 47.2% while 62.6% in the 40 μM ICS I administration group. Therefore, ICS I can induce G1 phase cell cycle arrest through IL-6/STAT3 pathway. Cyclin D1 and CDK4 are downstream target genes of STAT3, which have the ability to control the progression of the cell cycle from the G1 phase to the S phase [29, 30]. Overexpression of Cyclin D1 leads to dysregulation of CDK4 activity, which results in uncontrolled cell proliferation regulatory mechanisms and accelerated cell cycle progression [29]. Clinopathological studies have shown that overexpression of Cyclin D1 has been linked to poor prognosis in a wide variety of human malignancies [31]. Studies support the use of Cyclin D1 expression as a survival indicator for breast cancer patients [32]. To discover the underlying mechanism of ICS I disrupting the IL-6-induced breast cancer cell cycle, we investigated whether ICS I affects the expression of Cyclin D1 and CDK4. RT-qPCR confirmed this result, showing that ICS I downregulated IL-6-induced expression of Cyclin D1 and CDK4 (Fig. 4B and C). These data suggested that inhibition of STAT3 activation by ICS I induced cell cycle disruption and cell growth suppression.

Activation of STAT3 also brings about the abnormal expression of genes closely related to bcl-2 and bax [33]. Bcl-2 is unique among proto-oncogenes that prevent programmed cell death [34]. Bax is an apoptosis-promoting gene in the bcl-2 gene family and is utilized in conjunction with bcl-2 in tumor research [35]. Disruption of the STAT3 signaling pathway decreases the expression of the survival factor bcl-2 and activates the pro-apoptotic protein bax [36]. Apoptosis may be divided into three phases: the induction phase, the execution phase, and the degradation phase [37]. The induction phase is the process by which cells receive various apoptotic signals. The execution phase is the process by
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which apoptotic signals cause changes in intracellular signal transduction pathways and activate key signaling molecules. The degradation phase is the process by which activated hydrolases cleave intracellular substrates and caused changes in cell morphology and final cell lysis. Apoptosis is an extremely complex biological process, especially in mammalian cells, which is induced by specific signals and involves the collaboration of hundreds of genes. The genes involved in apoptosis often have multiple biological functions, for example, the bcl-2 not only causes apoptosis, but also participates in various processes such as cell cycle regulation, differentiation, and DNA damage repair. Bcl-2 has been previously reported to inhibit cell proliferation by delaying the G0/G1 to S phase transition [38–40]. In breast cancer cells, ICS I can reduce bcl-2 mRNA expression while increasing bax expression (Fig. 4D and E). Thus, ICS I is able to regulate the expression of bcl-2 and bax at concentrations that are not cytotoxic, possibly acting in the induction phase of apoptosis.

Figure 1. Epimedium flavonoids (EF) suppresses STAT3 phosphorylation, proliferation and migration in 4T1 cells. (A) After being exposed to EF for 1 h, 4T1 cells were subsequently activated with IL-6 at a concentration of 50 ng/mL for 12 h. Western blot analysis revealed that EF inhibited IL-6-induced p-STAT3 (Tyr705) in breast cancer cells. (B) Colony formation assays were performed in 4T1 cells after EF and IL-6 (100 ng/mL) treatment for 5 days. The area of colonies were counted by imageJ. (D) Cell migration was assessed by using Image J software. (E) After being exposed to EF for 1 h 4T1 cells were subsequently activated with IL-6 at a concentration of 100 ng/mL for 24 h, and were subjected to a scratch migration assay. All of the data are presented as means ± SEM from three biological samples. **P < .01, ***P < .001 vs. the control group. *P < .05, ***P < .01 vs. the IL-6 group. ns, non-significant vs. the IL-6 group.
The results indicated that ICS I can hinder the cell cycle and modulate Cyclin D1, CDK4, bcl-2, and bax to regulate proliferation and survival of breast cancer cells.

ICS I suppresses tumor growth and lung metastasis in the 4T1 breast cancer model probably through IL-6/STAT3 pathway

One of the models of breast cancer that is employed the most frequently involves the implantation of 4T1 cells into BALB/c mice [41–43]. When 4T1 cells are injected into the mammary gland of BALB/c female mice, spontaneous lung metastases develop. These metastases are analogous to what happens in clinical practice [44, 45]. Female BALB/c mice had their fourth pair of fat pads implanted with 4T1-luc cells. Mice were divided at random into three groups and given CMC-Na (vehicle), ICS I (25 mg/kg), and ICS I (50 mg/kg) intragastrically after 5 days (n = 6). Both the weight and volume of tumors were calculated. H&E staining, also known as hematoxylin and eosin, was utilized so that the morphology of tumor cells could be determined. In the vehicle group, tumor cells were arranged in a spoke-like pattern with obvious heterogeneity, and multinucleated tumor giant cells were seen. But, the tumor cells in the ICS I group were disorganized and necrotic lysis was seen in some areas (Fig. 5A). According to the results of our investigation, the progression of tumors was slowed down in mice treated with ICS I in comparison to animals treated with the vehicle control agent. Both tumor weight and tumor volume were considerably reduced in the ICS I-treated group compared to those in the vehicle group (Fig. 5B–D). IL-6 pathway can directly stimulate the proliferation, survival, and invasion of tumor cells [14]. Immunohistochemical analysis revealed that IL-6 expression in the vehicle group was higher than in the ICS I treatment group (Fig. 5E and F). In addition, we evaluated the impact of ICS I on the IL-6/STAT3 signaling pathways. As indicated in Fig. 6A–C, the therapy group had a considerable decrease in phosphorylated STAT3 levels. Following that, we examined the status of downstream targets in the IL-6/STAT3 pathway. The tumor tissue expressions of vimentin, cleaved caspase 3, bcl-2, bax, Cyclin D1, and CDK4 from different treated groups were detected. Our findings indicated that the expressions of vimentin, bcl-2, Cyclin D1, and CDK4 were decreased while the expressions of pro-apoptotic proteins bax and cleaved caspase 3 were increased in the ICS I-treated groups (Fig. 6A,D–J). The above data reflected ICS I suppressed tumor progression by inhibiting the IL-6/STAT3 pathway in vivo.

In vitro lung bioluminescence imaging revealed a substantial increase in lung metastasis in animals that had never received ICS I in the 4T1-luc mouse breast cancer model. Moreover, H&E staining showed inflammatory infiltration and hyperemia edema were observed in the interstitium of...
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**Figure 3.** ICS I suppresses proliferation and migration in 4T1 cells. (A) 4T1 cells were treated with or without ICS I and IL-6 (100 ng/mL) for 24 h, and were subjected to a scratch migration assay. (B) Cell migration was assessed by using ImageJ software. (C) The area of colonies were counted by ImageJ. (D) Colony formation assays were performed in 4T1 cells after ICS I and IL-6 (100 ng/mL) treatment for 5 days. (E) After being exposed to ICS I for 1 h, 4T1 cells were subsequently activated with IL-6 at a concentration of 50 ng/mL for 12 h. Western blot for vimentin protein level in 4T1, and β-actin was used as the loading control. Q-PCR analysis of MMP2 (F), MMP9 (G) and GAPDH mRNA expression in 4T1. All of the data are presented as mean ± SEM from three biological samples. *P < .05, **P < .01, ***P < .001 vs. the control group. #P < .05, ##P < .01, ###P < .001 vs. IL-6 group. ns, non-significant vs. the IL-6 group.
Figure 4. ICS I induces G1 phase cell cycle arrest and effects STAT3 downstream target genes in breast cancer cells. 4T1 cells were treated with or without IL-6 (50 ng/mL) and various concentrations of ICS I. (A) Cell cycle distribution of 4T1 cells were detected by flow cytometry after ICS I and IL-6 treatment. Quantitative real-time PCR analysis was used to detect the mRNA expression of Cyclin D1 (B), CDK4 (C), bcl-2 (D), bax (E), and GAPDH in 4T1. All data are presented as mean ± SEM from three biological samples. ## P < .01, ### P < .001 vs. the control group. * P < .05, ** P < .01 vs. IL-6 group. ns, non-significant vs. the IL-6 group.
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Figure 5. ICS I suppresses tumor growth in the 4T1 breast cancer model, causing tumor cell necrosis. The BALB/c mice bearing 4T1 cells were intragastrically administrated with CMC-Na (vehicle) or ICS I (25 or 50 mg/kg) for 27 days (n = 6). (A) The tumor sections were stained with hematoxylin and eosin (H&E, scale bars, 200 µm), and the above photos were representative of those section. (B) Representative images of the tumors. The scale bar represents 1 cm. The tumor weights (C) and tumor volumes (D) were measured. (E) The Immunohistochemistry (IHC) approach was utilized to analyze the levels of IL-6 expression that were found in tumor tissues. (F) Quantification of IL-6 in tumor tissues expression in different treatment group. All data are presented as mean ± SEM. *P < .05, **P < .01, ***P < .001 vs. the vehicle group. ns, non-significant vs. the vehicle group.
Figure 6. In vivo tumor models, ICS I inhibits IL6/STAT3 pathway and downstream target genes. (A) The expressions of bcl-2, p-STAT3 (Tyr705), and vimentin in tumor tissues were evaluated by IHC method. Quantification of p-STAT3 (Tyr705) (B), vimentin (C), and bcl-2 (D) in tumor tissues expression in different groups. Quantitative real-time PCR analysis of CDK4 (E), vimentin (F), bcl-2 (G), and GAPDH mRNA expression in tumor tissues. Western blot for cleaved caspase 3, bcl-2, bax (H), Cyclin D1, and CDK4 (I) protein levels in tumor tissues, and β-actin was used as the loading control (n = 6). All data are presented as means ± SEM. **P < .01, ***P < .001 vs. the vehicle group.
Figure 7. ICS I has effect on lung metastasis from breast cancer. Representative image of the lung section stained with H&E (scale bars, 200 µm) (A) and in vitro lung bioluminescence imaging analysis of vehicle and ICS I groups (B). (C) ROI (region of interest) of lungs were counted by Living Image 4.5.2. Quantitative real-time PCR analysis of MMP9 (D), vimentin (E) and GAPDH mRNA expression in lung tissues (n = 6). All data are presented as means ± SEM. *P < .05, **P < .001 vs. the vehicle group. ns, non-significant vs. the vehicle group.
Hou et al. observed lesion nodules in the lung in the vehicle group. In contrast, the ICS I therapy group showed reduced lesion nodules in the stroma, with obvious nuclear atypia and mitosis. Inflammatory cells were observed in the trachea, and some alveoli had merged. Meanwhile, lung metastasis and damage were decreased in the ICS I therapy group (Fig. 7A–C). RT-qPCR indicated a decrease in mRNA expression of MMP9 and vimentin (Fig. 7D and E), suggesting that ICS I could suppress lung metastasis from breast cancer. Through regulating the IL-6/STAT3 pathway and its downstream target genes, ICS I suppressed breast cancer development and metastasis, indicating a potential method for the therapeutic treatment of breast cancer.

Discussion

Cancer is a major leading cause of death worldwide [46]. Breast cancer is one of the three types of cancer that occur most frequently around the globe. The understanding of breast cancer has been progressively enhanced by the constant advancement of fundamental cancer science. In recent years, molecular targeted treatment has emerged as one of the most active research areas in breast cancer [47]. The present availability of innovative targeted medicines increases the likelihood of long-term control in metastatic breast cancer [48]. In the tumor microenvironment, IL-6/STAT3 signaling activation promotes tumor growth, survival, invasion, and metastasis while strongly inhibiting immune responses [49]. Therefore, targeting the IL-6/STAT3 signaling pathway is considered to be an important way to treat tumors [14].

With significant clinical efficacy and few adverse effects, traditional Chinese medicine (TCM) has long been used empirically for the treatment and prevention of breast cancer. In particular, TCM is used as an essential adjuvant treatment for patients with metastatic or advanced breast cancer in China and neighboring countries [50]. Due to its pharmacological properties in modulating immune function and anti-tumor activity, Epimedi Folium is a potential herbal medication for tumor immunotherapy [51]. ICS I is the isolated active component from Epimedi Folium. According to previous research, ICS I increased the proliferation and development of bone marrow hematopoietic cells and protected a damaged immune system by modulating hematopoietic cytokine levels, balancing bcl-2/bax, and suppressing caspase-3 expression [52]. ICS I also suppressed the development of B16F10 melanoma in vivo by modulating intestinal microbiota and upregulating several lymphocyte subsets [18]. No research has documented a therapeutic impact of ICS I on breast cancer yet. In our study, we found that ICS I could inhibit IL-6-induced STAT3 phosphorylation in breast cancer cells. Intriguingly, in vivo experiments also showed that ICS I possessed an anticancer impact via the IL-6/STAT3 pathway. These demonstrated that ICS I may be a new anti-breast cancer drug. Next, we observed that ICS I inhibited cell growth by stopping the G1 phase of cell cycle. Importantly, ICS I inhibited breast cancer migration. In vitro lung bioluminescent imaging and H&E staining showed that ICS I lessened lung metastasis and damage caused by breast cancer. It is possible that ICS I may open up new treatment possibilities for breast cancer. Therefore, this preclinical investigation gives a justification for ICS I to suppress IL-6/STAT3, which presents possibilities for enhancing the therapy of breast cancer in the future. This suggests that ICS I is a potential candidate for anti-breast cancer patients.

Icariin is the major active ingredient of Epimedi Folium. Icariin and its derivatives are mainly responsible for biological activities against anti-cardiovascular disease, anti-inflammation, and anti-tumor effects [53]. ICS I can be formed by the removal of rhamnose residues from Icariin, an isopentenylated flavonol glycoside. Up to date, there are few studies on the biological functions of ICS I, probably due to its low content in Epimedi Folium. However, the study found...
Icariin can be metabolized to ICS I in vivo [54]. It is possible that ICS I exerts its drug effect in vivo in this way. Moreover, recent study has shown that ICS I can be produced on a large scale by whole-cell catalysis, which solves one of the difficult problems in ICS I development [55]. Therefore, the pharmacological effects of ICS I are expected to be further developed. In this study, the antitumor effects of ICS I were enriched.

ICS I is an isopentenylation flavonol glycoside with glucose group and methoxy group. Flavonoids are found in plants with a broad spectrum of biological activities. In plants, most flavonoids exist in bound form which combine with sugars to be glycosides, and a small proportion exists in free form. Several in vitro studies have shown that flavonoids inhibit abnormal activation of the JAK-STAT pathway and have anti-inflammatory, antioxidant, anticancer, antidepressant, and antibacterial properties, which have outstanding medicinal value and are commonly used to prevent and treat cancer [56, 57]. flavonoids are classified according to different structural characteristics. Some flavonoids have similar pharmacological effects, which may be related to their chemical structures. Flavonol is a derivative of 2-phenylchromatin ketone-3-alcohol. Quercetin is also a natural flavonol compound that is widely found in buckwheat, hawthorn, and sea-buckthorn. Quercetin can synergistically enhance the anticancer efficacy of docetaxel through induction of apoptosis and modulation of JAK/STAT3 signaling pathways in MDA-MB-231 breast cancer cell [58]. flavones are compounds with 2-phenylchormone as the parent nucleus, including Apigenin and Luteolin. Apigenin [56] and Luteolin [59] can directly inhibit STAT3 phosphorylation in breast cancer cells. Thus, flavonoids with different structures may be able to exert effects on STAT3 activation in breast cancer cells.

Metastasis of vital organs is a major cause of high mortality from breast cancer, with more than 90% of breast cancer-related deaths associated with metastasis [60, 61]. Among these, pulmonary metastasis is alarming since it is associated with a 60–70% death rate [62]. Because breast cancer metastasis is a dynamic multi-step process with complex pathways and dispersed sites of occurrence, there are few viable treatment strategies that may effectively suppress breast cancer metastasis [63]. Tumor models are critical to our understanding of cancer and the development of cancer therapy. 4T1 murine breast cancer cell line is one of the most widely used breast cancer models [64]. 4T1 cells share significant molecular characteristics with human TNBC and are a common model for metastatic tumors [41]. In metastatic breast cancer, the hope of long-term disease control is gradually becoming an increasing reality, thanks to the promise that is held by novel targeted medicines. ICS I effectively suppressed lung metastasis from breast cancer. These findings point to the possibility that ICS I might be an effective drug for the treatment of 4T1 breast cancer metastasis. There are also some limitations to this study. First, there was no data on the effect of ICS I in the native tumor cell without IL-6 stimulation. Moreover, there was no control using the IL-6 antagonist to compare whether ICS I was indeed mediated through this pathway. Further drug target studies and clinical trials are needed to confirm its effectiveness in treating breast cancer metastasis.

Conclusion

This research is the first to indicate that ICS I has efficacy against breast cancer probably through inhibiting the IL-6/STAT3 pathway. The data suggested that ICS I has therapeutic and antimetastatic effects on breast cancer probably by inactivating the IL-6/STAT3 pathway (Fig. 8).

Supplementary Material

Supplementary data are available at Journal of Pharmacy and Pharmacology online.

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Author contributions

Manting Hou, Hui Li, Tingting He: investigation, validation, visualization, software, and original draft. Siwen Hui and Wenzhang Dai: investigation, formal analysis, and data curation. Xiaorong Hou: visualization and Jing Zhao methodology and formal analysis. Jia Zhao and Jincui Wen: investigation and software. Wen Kan: visualization and formal analysis. Xiaoyan Zhan, Zhaofang Bai, and Xiaohu Xiao: conceptualization, methodology, validation, re-sources, data curation, supervision, project administration, review & editing, and funding acquisition.

Conflict of interest statement

None declared.

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Data availability

Upon reasonable request, the corresponding author can provide the data that back up the study’s conclusions.

References

ICS I inhibits breast cancer


