**Scutellaria barbata** D. Don inhibits growth and induces apoptosis by suppressing IL-6-inducible STAT3 pathway activation in human colorectal cancer cells

QIQIN JIANG¹²*, QIONGYU LI¹*, HONGWEI CHEN¹, ALING SHEN¹, QIAOYAN CAI¹³, JIUMAO LIN¹³ and JUN PENG¹³

¹Academy of Integrative Medicine; ²People’s Hospital; ³Fujian Key Laboratory of Integrative Medicine on Geriatrics, Fujian University of Traditional Chinese Medicine, Fuzhou, Fujian 350122, P.R. China

Received September 9, 2014; Accepted August 4, 2015

DOI: 10.3892/etm.2015.2692

**Abstract.** One of the most critical cellular signal transduction pathways known to malfunction in colorectal cancer is the interleukin-6/signal transducer and activator of transcription 3 (IL-6/STAT3) pathway. *Scutellaria barbata* D. Don (SB) is well-known traditional medicine in China that targets STAT3 signaling, and it has long been used to treat various types of cancer; however, the precise mechanism of its anti-tumor activity remains largely unclear. In order to further elucidate this underlying mechanism, an ethanol extract of SB (EESB) in cancer treatment. The aim of the present study was to evaluate the effects of EESB on the IL-6-inducible STAT3 pathway. We tested the dose-response association between EESB, IL-6-induced proliferation and apoptosis using an MTT assay, colony formation and flow cytometry analysis in vitro. In addition, caspase-9 and caspase-3 activation was determined using a colorimetric assay, the activity of IL-6-induced STAT3 pathway was evaluated using western blot analysis, and the expression levels of cyclin D1, cyclin-dependent kinase 4, Bcl2 and Bcl2-associated X were determined using reverse transcription-polymerase chain reaction and western blot analysis. In the present study it was found that EESB could significantly inhibit the IL-6-mediated increase in STAT3 phosphorylation levels and transcriptional activity in HT-29 human colon carcinoma cells, resulting in the suppression of cell proliferation and the induction of apoptosis. In addition, treatment with EESB markedly inhibited the IL-6-induced upregulation of cyclin D1 and B-cell lymphoma-2, two key target genes of the STAT3 pathway. These results suggest that treatment with EESB could effectively inhibit the proliferation and promote the apoptosis of human colon carcinoma cells via modulation of the IL-6/STAT3 signaling pathway and its target genes.

**Introduction**

With an estimated annual incidence of >1 million new cases worldwide, colorectal cancer (CRC) is one of the most frequently occurring human malignant neoplasms. Approximately 1 in 3 individuals that develop CRC succumb to the disease (1). Studies have demonstrated that aberrant tyrosine phosphorylation, or activation of signal transducer and activator of transcription 3 (STAT3), acts as a regulator of tumorigenesis (2) and that the expression rates of STAT3 and phosphorylated- (p-)STAT3 in CRC tissues are significantly higher than those in adjacent normal intestinal mucosa tissues (3). Interleukin-6 (IL-6) is a proinflammatory cytokine that is primarily produced by the cells comprising the tumor microenvironment. Fibroblasts, myeloid cells and lymphoid cells. IL-6 plays a key role in promoting the proliferation and inhibition of apoptosis (4), as it binds to its receptor (soluble IL-6 receptor) and coreceptor (glycoprotein 130, or gp130), resulting in the activation of the associated Janus kinases (JAKs). Activated JAKs phosphorylate gp130, leading to the recruitment and activation of STAT3 (5). STAT3 is an important transcription factor that plays an essential role in cell survival and proliferation (6,7). It is known that the overexpression of cyclin D1 and B-cell lymphoma-2 (Bcl-2), among others, mediated by the abnormal activation of IL-6/STAT3, leads to excessive cell proliferation and apoptosis resistance. This, in turn, may cause tumorigenesis.

Inhibition of STAT3 transcriptional activity has been demonstrated to increase the rate of apoptosis in cancer cells (8). STAT3 has therefore been validated as a novel anti-cancer drug target, and targeting the STAT3 signaling pathway is considered a novel and promising therapeutic strategy in the

---

Correspondence to: Dr Jiumao Lin or Dr Jun Peng, Academy of Integrative Medicine, Fujian University of Traditional Chinese Medicine, 1 Qiuyang Road, Fuzhou, Fujian 350122, P.R. China

E-mail: jiumaolin@hotmail.com

E-mail: pjunlab@hotmail.com

*Contributed equally

**Key words:** *Scutellaria barbata* D. Don, colorectal cancer, interleukin-6/signal transducer and activator of transcription 3 pathway, apoptosis, proliferation
treatment of cancer (9). Despite advances in chemotherapy, a regimen of 5-fluorouracil, in combination with oxaliplatin and irinotecan, remains one of the most important treatments of CRC (10); however, the majority of patients with CRC develop drug resistance and fall subject to metastasis. This problem has resulted in an increased interest in natural medicines, with studies in cancer therapeutics revisiting traditional herbal medicines. A number of herbal extracts or mixtures based on traditional medicines have exhibited anticancer effects with fewer or no side effects as compared with other anticancer therapeutics, including chemical compounds and targeting antibodies (11-13).

Scutellaria barbata D. Don (SB) is an important component of numerous medicinal formulas that have traditionally been used in China to treat a range of types of cancer, including CRC. We have recently reported that ethanol extract of SB (EESB) can exert numerous effects: i) Induction of cancer cell apoptosis by activating the mitochondrial-dependent pathway; ii) inhibition of tumor angiogenesis via suppression of Hedgehog signaling; and iii) induction of G1/S arrest in human colon carcinoma cells by modulating a number of signaling pathways associated with the cell cycle (14-17). In order to further elucidate the mechanism underlying the tumoricidal activity of EESB, the aim of the present study was to explore its effects on the IL-6-mediated activity in HT-29 human carcinoma cells, including cell proliferation and apoptosis, the STAT3 phosphorylation level and transcriptional activity, and the expression of a number of target genes of the IL-6/STAT3 signaling pathway.

Materials and methods

**Materials and reagents.** Dulbecco’s modified Eagle's medium (DMEM), fetal bovine serum (FBS), penicillin-streptomycin, trypsin-EDTA, TRIzol® reagent, and caspase-9 and caspase-3 activation kits were purchased from Invitrogen (Life Technologies, Carlsbad, CA, USA). Monoclonal antibodies against Bcl-2, Bcl2-associated X protein (Bax), cyclin D1 and cyclin-dependent kinase 4 (CDK4) and horseradish peroxidase (HRP)-conjugated secondary antibodies were obtained from Cell Signaling Technology, Inc. (Beverly, MA, USA). SuperScript™ II reverse transcriptase was obtained from Promega Corp. (Madison, WI, USA). A bicinechonic acid (BCA) protein assay kit was purchased from TianGen Biotech (Beijing) Co., Ltd. (Beijing, China). Unless otherwise stated, all other chemicals were obtained from Sigma Chemical Co. (St. Louis, MO, USA).

**Preparation of EESB.** The original herb was collected in the Henan region of China and was identified as SB by Dr Wei Xu at the Department of Pharmacology, Fujian University of Traditional Chinese Medicine (Fuzhou, China). The plants were dried and cut into small pieces, and EESB was prepared as described in a previous study (16). Stock solutions of EESB were prepared by dissolving the EESB powder in 40% dimethyl sulfoxide (DMSO) to a concentration of 500 mg/ml, and the solutions were stored at -20°C. The working concentrations of EESB were made by diluting the stock solution in the culture medium. The final concentrations of DMSO in the medium were <0.5%.

**Cell culture.** HT-29 human colon carcinoma cells were obtained from the Cell Bank of the Chinese Academy of Sciences (Shanghai, China). The cells were grown as an adherent monolayer in DMEM culture media containing 10% v/v FBS, 100 U/ml penicillin and 100 µg/ml streptomycin in a humidified incubator at 37°C with 5% CO₂.

**EESB and IL-6 treatment.** HT-29 cells were cultured with DMEM containing 10% FBS and 1% penicillin/streptomycin. When the cells reached 50% confluence, the complete medium was changed with FBS-free medium overnight. The cells were then pretreated with various concentrations of EESB in complete DMEM for 1 h, followed by stimulation with 10 ng/ml IL-6 (Sigma Chemical Co.) for the indicated periods.

**Evaluation of cell viability by MTT assay.** The cells were harvested and re-suspended at a final concentration of 1x10⁵ cells/ml and were seeded into a 96-well plate at 100 µl/well. Following incubation for 24 h at 37°C, the cells were treated with different concentrations of EESB and/or IL-6 for another 24 h. Subsequently, 100 µl MTT (0.5 mg/ml) was added to each well. The plates were incubated at 37°C for 4 h, and 100 µl DMSO was added to dissolve the purple formazan crystals. The absorbance was then read at 570 nm with an ELISA reader (Model ELx800; BioTek Instruments, Inc., Winooski, VT, USA).

**Observation of morphological changes.** HT-29 cells were seeded into six-well plates in 2 ml medium at a density of 2.5x10⁵ cells/well. The cells were treated with various concentrations of EESB and/or IL-6 for 24 h. The cell morphology was observed using a phase-contrast microscope (Leica, Solms, Germany). Images were captured at a magnification of x200.

**Colony formation.** HT-29 cells from the exponentially growing cultures were seeded into 12-well culture plates at a density of 1x10⁵ cells/well and treated with different concentrations of EESB and/or IL-6 for 24 h. The cells were then harvested and seeded into six-well plates at a final concentration of 1x10³ cells/well in 2 ml fresh medium. Following incubation for 8 days in a humidified incubator at 37°C with 5% CO₂, the formed colonies were fixed in MeOH-HAc (3:1, v/v) for 10 min, stained with crystal violet and counted. Cell survival was calculated by normalizing the survival of the control cells as 100%.

**Cell cycle analysis.** A total of 2.5x10⁵ HT-29 cells were seeded into six-well plates in 2 ml medium and treated with the indicated concentrations of EESB and/or IL-6 for 24 h. The cells were harvested and adjusted to a concentration of 2x10⁵ cells/ml. The HT-29 cell cycle progression was determined through flow cytometric analysis using a propidium iodide (PI) staining cell cycle assay kit (BD Biosciences, Franklin Lakes, NJ, USA). The cells were fixed in 70% ethanol at 4°C overnight. The fixed cells were washed twice with cold phosphate-buffered saline (PBS) and then incubated for 30 min with ribonuclease (8 µg/ml) and PI (10 µg/ml). The fluorescent signal was detected through the FL2 channel, and the proportion of DNA in various phases was analyzed using
Detection of apoptosis through flow cytometric analysis with Annexin V/PI staining. A total of 2x10^5 HT-29 cells were seeded into six-well plates in 2 ml medium and treated with the indicated concentrations of EESB and/or IL-6 for 24 h. The apoptosis of the HT-29 cells was then determined through flow cytometric analysis using a fluorescence-activated cell sorter (FACSCalibur™; BD Biosciences) and an Annexin V-fluorescein isothiocyanate/PI kit (KeyGen Biotech, Nanjing, China). Staining was performed according to the manufacturer's instructions. In this assay, the Annexin V/PI double-negative population indicated viable cells, and the Annexin V-positive/PI-negative or Annexin V/PI double-positive populations represented cells undergoing early or late apoptosis, respectively.

Analysis of caspase-9/caspase-3 activation. Caspase-9 and caspase-3 activity was determined via a colorimetric assay using caspase-9 and caspase-3 activation kits, following the manufacturer's instructions (Invitrogen). Following treatment with various EESB concentrations and 10 ng/ml IL-6 for 24 h, the HT-29 cells were lysed with the provided lysis buffer for 30 min on ice. The lysed cells were then centrifuged at 16,000 x g for 10 min. The protein concentration of the clarified supernate was determined, and 100 µg protein was incubated with 50 µl of the colorimetric tetrapeptide Leu-Glu-His-Asp-p-nitroaniline (pNA), a specific substrate of caspase-9, or with Asp-Glu-Val-Asp-pNA, a specific substrate of caspase-3, at 37˚C in the dark for 2 h. Samples were read at 405 nm in the ELISA reader. The data were normalized to the activity of the caspases in the control cells (treated with PBS vehicle) and were represented as the fold of control.

Reverse transcription polymerase chain reaction (RT-PCR) analysis. A total of 2x10^5 HT-29 cells were seeded into six-well plates in 2 ml medium and treated with the indicated concentrations of EESB and/or IL-6 for 24 h. The total RNA was isolated with TRIZol reagent. Oligo(dT)-primed RNA (1 µg) was reverse-transcribed with SuperScript II reverse transcriptase according to the manufacturer's instructions (Promega Corp.). The obtained cDNA was used to determine the mRNA expression level of cyclin D1, (forward, 5'-TGG ATG CTG GAG GTC TGC GAG GAA-3' and reverse, 5'-GGC TTC GAT CTG CTC GCA GGC GGC-3', at 57˚C), CDK4 (forward, 5'-CAT GTA GAC GAG GTC GCA GGC-3' and reverse, 5'-AAC TGG CGC ATC AGA TCC TAG-3', at 58˚C), Bcl-2 (forward, 5'-CAG CTG CAC CTG ACG CCGT-3 and reverse, 5'-GCC TCC GGT ATC CTT GAT CC-3', at 55˚C) and Bax (forward, 5'-TGC TTC AGG GTT TCA TCC AGG-3' and reverse, 5'-TGGCAA AGT AGA AAA GGG CGA-3', at 55˚C) by PCR analysis. GAPDH (forward, 5'-GT CAT CCA TGA CAA CTT TGG-3' and reverse, 5'-GA GCT TGA CAA AGT GGT CCGT-3' at 58˚C) was used as a reference gene. The PCR cycling conditions for all sequences were as follows: 35 Cycles of denaturation at 95˚C for 3 min, annealing for 40 sec, and extension for 45 sec, and a final extension at 72˚C for 10 min. Samples were analyzed using gel electrophoresis (1.5% agarose). The DNA bands were

An MTT assay was used to determine the effect of EESB on HT-29 cell viability

Western blot analysis. HT-29 cells (2x10^5/ml) were seeded into flasks and treated with the indicated concentrations of EESB and/or IL-6 for 24 h. The treated cells were lysed with mammalian cell lysis buffer containing different protein inhibitors. The total protein concentrations were determined via BCA assay. Equal quantities of protein from each cell lysate were subjected to SDS-PAGE and transferred onto polyvinylidene difluoride membranes. The membranes were blocked with 5% nonfat dry milk for 2 h and incubated with the desired primary antibody directed against p-STAT3, STAT3, cyclin D1, CDK4, Bcl-2, Bax and β-actin, at dilutions of 1:1,000, overnight at 4˚C. To image the antibody-detected proteins, the appropriate HRP-conjugated secondary antibodies with chemiluminescence detection were used.

Statistical analysis. All data are the means of three determinations and were analyzed using the SPSS package for Windows (version 17.0; SPSS, Inc., Chicago, IL, USA). Statistical analysis of the data was performed with a Student's t-test and analysis of variance. P<0.05 was considered to indicate a statistically significant difference.

Results

EESB inhibits the growth of HT-29 cells. An MTT assay was used to determine the effect of EESB on HT-29 cell viability
in the presence of IL-6. As shown in Fig. 1A, IL-6 stimulation increased the viability of HT-29 cells to 167.89% compared with the control cells (P<0.05). Treatment with 0.5 to 2.5 mg/ml EESB for 24 h reduced the cell viability of the IL-6-stimulated cells from 88.06 to 61.72% (P<0.05). Furthermore, the effect of EESB on HT-29 cell survival was examined using a colony formation assay. As shown in Fig. 1B, treatment with 0.5, 1.5 and 2.5 mg/ml EESB for 24 h could reduce the survival rate of IL-6-stimulated cells by 20.35, 68.40 and 83.55% (P<0.05), respectively. To further verify these results, the effect of EESB on HT-29 cell morphology was evaluated via phase-contrast microscopy, since the morphology of cells in culture is indicative of the healthy status of the cells. As shown in Fig. 2, it was found that EESB treatment dose-dependently reduced the density of the HT-29 cells. Taken together, these data demonstrate that EESB inhibits the growth of IL-6-stimulated HT-29 cells.

**EESB blocks G1/S progression of HT-29 cells.** G1/S transition is one of the two main checkpoints used by a cell to regulate the cell cycle progress and thus the proliferation of the cell. The effect of EESB on the G1 to S progression in HT-29 cells was therefore investigated via PI staining, followed by fluorescence-activated cell sorting analysis. As shown in Fig. 3, the percentage proportion of S-phase cells was 34.10% for the untreated control; 36.02% for the IL-6-stimulated HT-29 cells; and 28.17, 17.28 and 11.79% for the IL-6-stimulated HT-29 cells treated with various EESB concentrations (0.5, 1.5 and 2.5 mg/ml, respectively) (P<0.05). These results indicate that EESB inhibits the proliferation of HT-29 cells by blocking the G1- to S-phase progression of the cell cycle.

**EESB induces apoptosis in HT-29 cells.** To determine whether EESB could induce cell apoptosis, Annexin V/PI staining...
with fluorescence-activated cell sorting analysis was used to examine the apoptosis of the HT-29 cells. As shown in Fig. 4A and B, the apoptosis of the HT-29 cells with stimulation of 10 ng/ml IL-6 was not significantly decreased compared with that of the control cells, which were not stimulated with IL-6 or EESB treatment (P>0.05); however, EESB treatment increased the percentage of cells undergoing apoptosis in a dose-dependent fashion (P<0.05, as compared with the cells stimulated with IL-6 but not treated with EESB). In the FACS diagram, early apoptosis is shown in the lower-right quadrant and late apoptosis in the upper right.

**EESB induces the activation of caspase-9 and caspase-3 in HT-29 cells.** The activation of caspase-9 and caspase-3 was examined via colorimetric assay using a specific chromophore. Caspases, the cytoplasmic aspartate-specific cysteine proteases, are the key proteins in the apoptotic response. Caspase-9 can activate caspase-3 so that the specific and vital cellular proteins are targeted and degraded. Subsequently, the nuclear DNA degrades and the apoptotic death of the cells occurs; therefore, the activation of caspases is important to the execution of apoptosis (14). As shown in Fig. 5, EESB treatment significantly and dose-dependently induced the activation of caspase-9 and caspase-3.
both caspase-9 and caspase-3 in the HT-29 cells (P<0.01, as compared with the cells stimulated with IL-6 but not treated with EESB). Additionally, IL-6 significantly inhibited the activation of caspase-9 and caspase-3.

**Discussion**

CRC is a complex and heterogeneous condition that is associated with several cellular signal transduction pathways (20-23). As a result of this, the use of specific inhibitors that only target a single pathway in complex tumor systems may prove ineffective. Furthermore, the long-term administration of numerous single-target agents can lead to the development of drug resistance and unwanted side effects (24). SB is a major constituent of numerous traditional medicinal formulas in China that are used to treat a range of types of cancer, including CRC. Several traditional herbal medicines are composed of multiple natural compounds, including SB. Herbal medicines are thus considered to be multi-component and multi-target agents that exert their therapeutic functions in a more holistic manner (25).

Numerous studies have shown that STAT3 is constitutively activated in a diverse range of human tumors (15,18). Furthermore, it has been suggested that aberrant STAT3 signaling can promote the initiation and progression of human cancer through either the inhibition of apoptosis or the induction of cell proliferation, angiogenesis, invasion and metastasis. The suppression of STAT3 activation is associated with the induction of tumor cell apoptosis (26). IL-6/STAT3 signaling regulates the survival and proliferation of intestinal epithelial cells and plays an important role in the pathogenesis of inflammatory bowel disease and CRC. The activation of the intracellular JAK/STAT3 signaling pathway, triggered by IL-6, leads to the induction of genes involved in the development of CRC (27). Cell proliferation is governed by the cell cycle, an order of events that is tightly regulated by a number of serine/threonine protein kinases known as CDKs and cyclins, such as CDK4 and cyclin D1 (28). Furthermore, Bax, Bcl2-associated X protein; CDK4, cyclin-dependent kinase 4; RT-PCR, reverse transcription polymerase chain reaction.

**Figure 7. Effect of EESB on the expression of cyclin D1, CDK4, Bcl-2 and Bax.** Cells were pretreated with various EESB concentrations for 1 h, followed by stimulation with 10 ng/ml IL-6 for 24 h. (A) mRNA levels of cyclin D1, CDK4, Bcl-2 and Bax were determined using RT-PCR. (B) Protein expression levels of cyclin D1, CDK4, Bcl-2 and Bax were determined using western blot analysis. GAPDH and β-actin were used as the internal controls for the RT-PCR and western blot assay, respectively. EESB, EESB, ethanol extract of *Scutellaria barbata* D. Don; IL-6, interleukin-6; Bcl-2, B-cell lymphoma-2; Bax, Bcl2-associated X protein; CDK4, cyclin-dependent kinase 4; RT-PCR, reverse transcription polymerase chain reaction.
EEK treatment significantly inhibited the mRNA and protein expression of Bcl-2, cyclin D1 and CDK4. The EESB-induced inhibition of the IL-6-mediated STAT3 activation and of cyclin D1, CDK4 and Bcl-2 expression led to the suppression of HT-29 cell proliferation and the induction of cell apoptosis. Furthermore, EESB treatment increased the expression of Bax.

In conclusion, the results of the present study have shown that EESB can effectively inhibit the proliferation and promote the apoptosis of human colon carcinoma cells by modulating the IL-6/STAT3 signaling pathway and its target genes. Further studies should be performed in order to fully elucidate the molecular mechanism underlying the action of EESB in cancer treatment and to enable the development of more effective multi-target drugs for cancer therapy.

Acknowledgements

This study was sponsored by the Developmental Fund of Chen Keji Integrative Medicine (grant nos. CKJ2014013 and CKJ2015007), the Natural Science Foundation of Fujian Province of China (grant no. 2013J01333) and the Research Foundation of Fujian University of Traditional Chinese Medicine (grant no. X2012012).

References