Scutellaria barbata D. Don polysaccharides inhibit the growth of Calu-3 xenograft tumors via suppression of the HER2 pathway and angiogenesis

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Abstract. *Scutellaria barbata* D. Don, a perennial herb belonging to the family Lamiaceae, is widely distributed throughout China and the Republic of Korea, and has been traditionally used in folk medicine as an antitumor and anti-inflammatory agent. Polysaccharides isolated from *Scutellaria barbata* D. Don (PSB), have been reported to possess antitumor effects. However, the detailed antitumor mechanisms behind the effects of PSB remain unclear. In the present study, a non-small cell lung cancer cell line harboring the *HER2* gene mutation Calu-3, the Calu-3 cell line, was used to investigate the underlying mechanisms of the antitumor effects of PSB. The results revealed that PSB potently inhibited cell proliferation and human epidermal growth factor receptor (HER)2 phosphorylation *in vitro*, and also downregulated the expression of the downstream signaling molecules, including phosphorylated (phospho-)Akt and phospho-extracellular signal-related kinase. *In vivo*, PSB demonstrated efficacy at well-tolerated doses, including significant antitumor activity in a Calu-3 subcutaneous xenograft model. Immunohistochemistry (IHC) analysis revealed a PSB dose-dependent reduction of microvessel density, demonstrated by cluster of differentiation 31 staining. The present findings suggest that inhibition of tumor angiogenesis via suppression of the HER2 pathway may be one of the mechanisms by which PSB can be effective in the treatment of cancers.

Introduction

The human epidermal growth factor receptor (HER) family consists of HER1, HER2, HER3 and HER4. The HER proteins form hetero- and homodimers that activate signal transduction pathways to regulate numerous cellular processes, including the growth, proliferation and survival of cells (1). Therefore, the HER proteins have been considered to be involved in oncogenesis (2). Tumors that overexpress the HER2 protein or exhibit amplification of the *HER2* gene, in particular, are associated with aggressive disease progression and a poor prognosis (3,4). HER2 receptor overexpression occurs in 11-32% of non-small cell lung cancer (NSCLC) tumors, with an increased gene copy number documented in 2-23% of cases (5).

Worldwide, lung carcinoma is one of the leading causes of cancer-associated mortality in males and females (6). NSCLC accounts for ≤80% of all lung cancer cases. Patients have typically developed advanced disease by the time of diagnosis. The prognosis of patients with advanced lung cancer remains poor, and previous studies have indicated that conventional therapies may have reached a therapeutic plateau, as demonstrated by the five-year survival rate for NSCLC patients, which remains at 15% (7,8). These issues highlight the urgent requirement for the development of novel cancer chemotherapies. Recently, natural products, including those used in Traditional Chinese Medicine (TCM), have received interest as they result in relatively few side-effects and have been used clinically as alternative remedies for a variety of diseases for thousands of years (9,10). *Scutellaria barbata* D. Don, a perennial herb belonging to the family Lamiaceae, is widely distributed throughout China and the Republic of Korea, and has been traditionally used in folk medicine as an antitumor and anti-inflammatory agent (11,12). It has been reported that polysaccharides isolated from *Scutellaria barbata* D. Don (PSB) possess antitumor activity that suppresses the growth of numerous types of cancer *in vitro* and *in vivo* (13,14). To further elucidate the mechanism of the antitumor activity of PSB, the present study investigated its antitumor activity *in vivo* as well as the inhibitory effect of PSB on the HER2 pathway.

Materials and methods

Drug isolation and cell line. Scutellaria barbata D. Don was purchased from the Zhengzhou Market of Traditional Chinese Herbs (Chengdong Road, Zhengzhou, China) and was identified by Professor Yunzhi Du of Henan University of Traditional Chinese Medicine (Zhengzhou, Henan, China). PSB was prepared using the method described previously by Song *et al* (15). The dried and crushed *Scutellaria barbata*

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D. Don was extracted four times by decoction at 90˚C using deionized water, with each extraction period lasting 2 h. Subsequent to filtration, the resulting extract was mixed with four volumes of dehydrated ethanol, with the ethanol final concentration being 80%, and incubated overnight at 4˚C in a refrigerator. Subsequently, the mixture was centrifuged at 3,618 x g for 10 min, washed four times using dehydrated ethanol, and the precipitate was collected as crude PSB. Diethylaminoethyl-52 column chromatography (Whatman International Ltd., Maidstone, UK) was then used to purify the crude PSB, using NaCl solution at a concentration of 0.2 mol/l.

The Calu-3 cell line is a lung adenocarcinoma cell line in which *HER2* is amplified to an average copy number of 30 (16). The cells were obtained from the American Type Culture Collection (Manassas, VA, USA) and cultured in RPMI-1640 medium supplemented with 10% fetal bovine serum (FBS). The cells were maintained at 37˚C in a 5% CO_2 humidified atmosphere. The RPMI-1640 medium and FBS were purchased from Invitrogen (Carlsbad, CA, USA). Trastuzumab (Herceptin; Genentech, Inc., San Francisco, CA, USA) was used as a positive control.

Cell growth inhibition assay. Cell growth inhibition was measured using the MTT dye reduction method. The Calu-3 cells in RPMI-1640 medium supplemented with 10% FBS were plated at a density of $1x10^4$ cells/100 ml/well into 96-well plates and cultured with 10, 20, 40 and 80 μg/ml of PSB or 10 μg/ml Herceptin for 72 h, followed by the addition of 50 ml MTT solution (2 mg/ml; Sigma-Aldrich, St. Louis, MO, USA) to each well and further incubation for 2 h. The medium was removed, and the dark blue crystals in each well were dissolved in 100 ml dimethyl sulfoxide (Sigma-Aldrich). The percentage of cell growth inhibition was calculated as follows:

$$\text{Cell growth inhibition (\%)} = \frac{A_{492\ \text{vehicle}} - A_{492\ \text{treatment}}}{A_{\text{vehicle}492}} \times 100$$

where $A_{492\ \text{vehicle}}$ was the absorbance of the vehicle treatment [treated with phosphate buffered saline (PBS)] at 492 nm and $A_{492\ \text{treatment}}$ was the absorbance of the experimental group (treated with PSB or Heceptin) at 492 nm.

Western blot analysis. The Calu-3 cells were treated with various concentrations of PSB for 24 h, and the cells were then lysed in cell lysis buffer containing phosphatase and proteinase inhibitor cocktails (Sigma-Aldrich). The protein concentrations were determined using a bicinchoninic acid protein assay kit (Pierce Biotechnology, Rockford, IL, USA). Equal amounts of protein (50 μg) were separated by SDS/PAGE on 8% gels, blotted on polyvinylidene difluoride membranes (Bio-Rad Laboratories, Hercules, CA, USA), and probed with phosphorylated (p-)HER2, p-Akt, p-extracellular signal-regulated kinase (ERK), HER2, Akt and ERK rabbit monoclonal antibodies (catalog nos. 9923, 8609, 9271, 9272, 9101 and 9101, respectively; all purchased from Cell Signaling Technology, Danvers, MA, USA; dilution, 1:1000 with PBS), and subsequently with goat anti-rabbit horseradish peroxidase-conjugated secondary antibody, which was detected by chemiluminescence. The antibodies were purchased from Santa Cruz Biotechnology, Inc. (Dallas, TX, USA).

In vivo studies. Athymic BALB/c nu/nu male mice with an initial body weight of 20-22 g were obtained from Shanghai Laboratory Animal Center Laboratory Animal Co., Ltd. (Chinese Academy of Sciences, Shanghai, China) and housed under pathogen-free conditions with a 12 h light/dark cycle. Food and water were provided *ad libitum* throughout the experiment. In total, $5x10^6$ Calu-3 cells per mouse were injected subcutaneously into mouse flanks. The tumor volumes were measured in two dimensions, the length and width, using calipers prior to treatment and twice a week once the treatment was initiated. The mice were also weighed at these times. The tumor sizes were calculated using the standard formula, as follows: tumor size = (length x width^2) / 2. Mice that developed tumors 150-200 mm^3 in size were randomly allocated into the following four treatment groups, with eight mice in each group: Control, normal saline administered intraperitoneally once daily; 30 mg/kg Herceptin administered intraperitoneally twice a week; 100 mg/kg PSB administered intraperitoneally once daily; and 200 mg/kg PSB administered intraperitoneally once daily. The treatment regimen continued for 21 days and the mice were sacrificed 3 h subsequent to the last treatment. The tumors were excised and fixed overnight in 4% formalin, at 4˚C. The samples were then embedded in paraffin for immunohistochemical analysis. All animal experiments were performed in accordance with protocols approved by the Experimental Animal Center of the Henan University of Traditional Chinese Medicine Animal Care and Use Committee.

Immunohistochemistry analysis. The tumor specimens were fixed in 4% formalin for 24 h prior to being transferred to 70% ethanol. The tumor samples were subsequently embedded in paraffin, and 4-μm thick sections were cut and baked at 58˚C overnight, then placed onto microscope slides. The slides were successively incubated with the primary and secondary antibodies described in the western blot analysis section as wel as primary CD31 monoclonal antibody (catalog no. 3528; Cell Signaling Technology; dilution, 1:1000 with PBS), and visualized using a colorimetric method (DAB kit; Envision-HRP, Dako North America, Inc., Carpentaria, CA, USA). All immunostained sections were counterstained using hematoxylin. An automated Ventana Discovery XT Staining Module (Ventana Medical Systems, Inc., Tucson, AZ, USA) was used to conduct histological staining. The stained sections were analyzed using an Olympus BX60 microscope (Olympus Corporation, Tokyo, Japan) and quantitative analysis of section staining was performed using the Automated Cellular Imaging System (Clarient, Inc., Aliso Viejo, CA, USA). For the quantification of vascular area in lung tumors, up to four random fields for each tumor section at x100 magnification (60% center field) were captured subsequent to staining with anti-cluster of differentiation (CD)31 antibody. The vascular area was calculated using Image Pro software (Media Cybernetics, Warrendale, PA, USA).

Statistical analysis. All data were reported as the mean ± standard deviation for the indicated number of independently

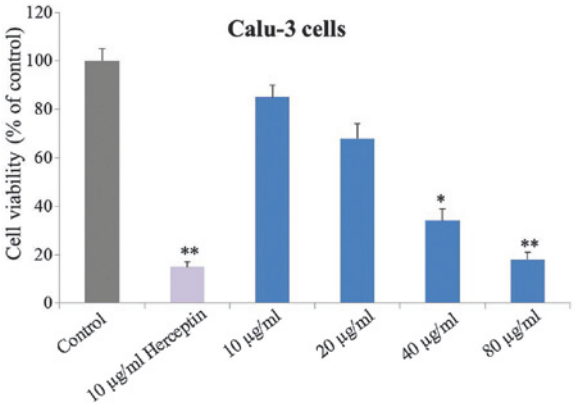

Figure 1. PSB dose-dependently inhibited Calu-3 cell growth. The Calu-3 cells were treated with 10, 20, 40 and 80 μg/ml PSB for 72 h. For the positive control, 10 μg/ml Herceptin was administered. The results are presented as the mean ± standard deviation. n=3; *P<0.05 vs. control group; **P<0.01 vs. control group. PSB, polysaccharides isolated from *Scutellaria barbata* D. Don.

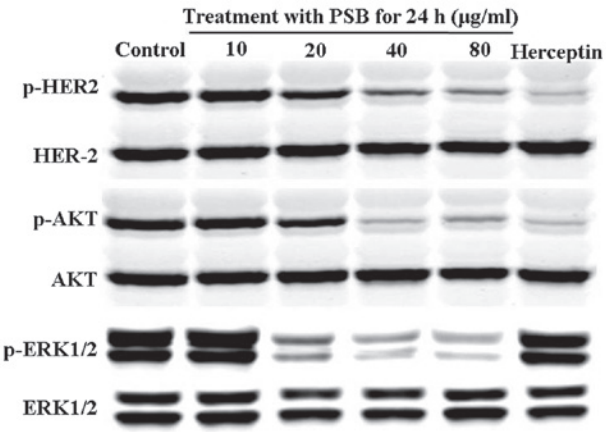

Figure 2. PSB inhibited the expression of p-HER2, p-Akt and p-ERK in Calu-3 cells. The Calu-3 cells were treated with 10, 20, 40 and 80 μg/ml PSB for 24 h. The positive control was performed using 10 μg/ml Herceptin. PSB, polysaccharides isolated from *Scutellaria barbata* D. Don; HER2, human epidermal growth factor receptor 2; p-HER2, phosphorylated HER2; ERK1/2, extracellular signal-regulated kinase 1/2; p-ERK1/2, phosphorylated ERK1/2; p-Akt, phosphorylated Akt.

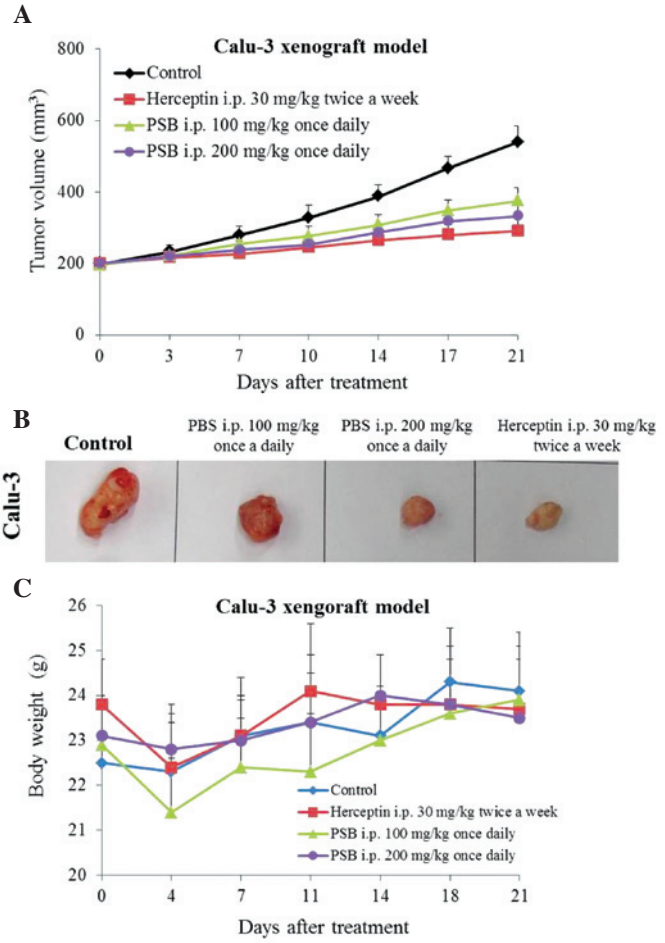

Figure 3. PSB inhibited tumor growth in a Calu-3 xenograft model. (A) Nude Calu-3 tumor-bearing mice were intraperitoneally injected once daily with PSB at the indicated dose or with the control vehicle for ≤21 days. The tumor volume was measured on the indicated days using calipers to yield the mean tumor volume. (B) The Calu-3 cell tumors were resected from nude mice on the last day of treatment. (C) Body weight changes during the process of efficacy study. Treatment with 100 and 200 mg/kg PSB demonstrated no toxicity in the Calu-3 xenograft model. For the positive control, 30 mg/kg Herceptin was administered. The data are presented as the mean ± standard deviated (n=8). *P<0.05 and **P<0.01 vs. control group. PSB, polysaccharides isolated from *Scutellaria barbata* D. Don; i.p. intraperitoneally administered.

performed experiments. The statistical analysis was carried out using Student's *t*-test and P<0.05 was considered to indicate a statistically significant difference.

Results

PSB-induced inhibition of proliferation in Calu-3 cells. The incubation of Calu-3 cells with 10, 20, 40 or 80 μg/ml PSB for 72 h resulted in a significant increase in the inhibition of cell proliferation, demonstrating that PSB-induced Calu-3 cell death occurred in a dose-dependent manner. The positive control treatment, consisting of the administration of 10 μg/ml Herceptin, also demonstrated a potent inhibitory effect on Calu-3 cell growth (Fig. 1).

PSB suppression of the HER2 signaling pathway in Calu-3 cells. In order to ascertain whether the expression of p-HER2 in Calu-3 cells would be affected by PSB, the phosphorylation states of HER2, Akt and ERK were assessed subsequent to 24 h of treatment with increasing doses of PSB, comprising 10, 20, 40 and 80 μg/ml PSB.

The present results indicated that the expression of p-HER2 in the Calu-3 cells treated with PSB for 24 h was down-regulated in a concentration-dependent manner compared with the vehicle group. High-dose PSB administration (80 μg/ml) was also indicated to completely inhibit p-HER2 expression. The expression of key downstream proteins, including p-Akt and p-ERK, was detected in Calu-3 cells, but PSB was found to demonstrate a concentration-dependent inhibition of the expression of p-Akt and p-ERK compared with the control group. The positive control of 10 μg/ml Herceptin was able to significantly inhibit the expression of p-HER and p-Akt, but only demonstrated a mild inhibitory effect on the expression of p-ERK (Fig. 2).

PSB inhibited tumor growth in the Calu-3 xenograft model. The *in vivo* efficacy of PSB against tumor growth was

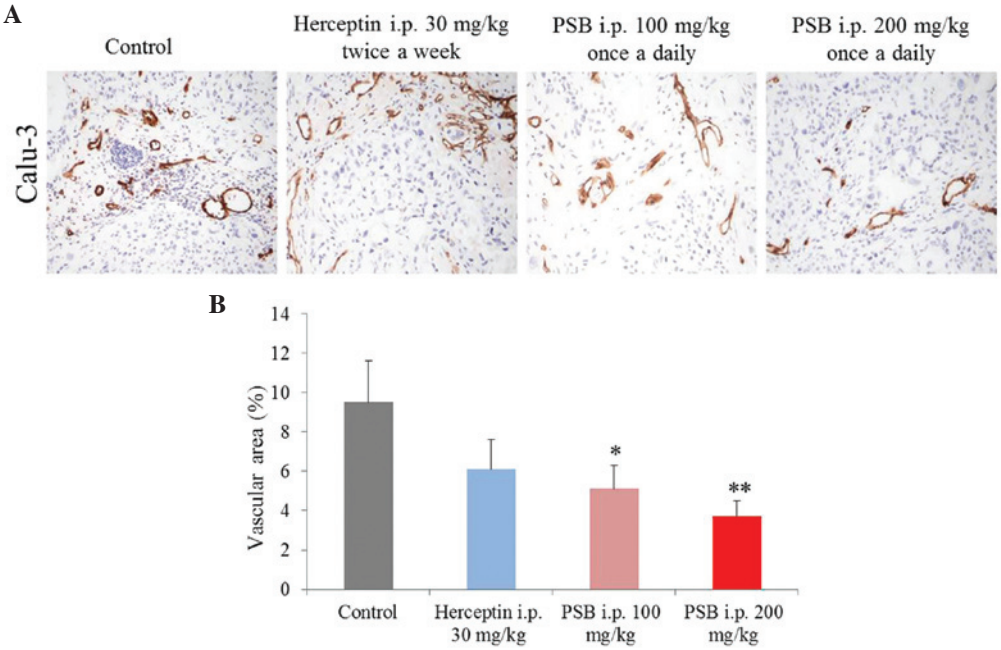

Figure 4. PSB significantly inhibited the expression of CD31 in a Calu-3 xenograft model. (A) Fresh tumors from each group were collected 3 h subsequent to the last treatment with 100 or 200 mg/kg PSB, and two days subsequent to the last treatment with 30 mg/kg Herceptin. (B) Representative CD31 staining of lung tumors (brown), viewed under a x100 magnification, 60% center field and vascular area (area of CD31-positive objects per field area x100%) are shown. *P<0.05 vs. control group. **P<0.01 vs. control group. PSB, polysaccharides isolated from *Scutellaria barbata* D. Don; CD31, cluster of differentiation 31; i.p., intraperitoneally administered.

investigated by evaluating its effect on tumor volume in a Calu-3 xenograft model, and the adverse effects of PSB were determined by measuring the body weight gain. As shown in Fig. 3A and B, the administration of PSB significantly decreased tumor volume in a dose-dependent manner compared with the control group (P<0.05). However, the positive control Herceptin exerted a more potent inhibitory effect on tumor volume in the Calu-3 xenograft model. Throughout the duration of the efficacy study, there was no bodyweight loss observed in any of the groups (Fig. 3C).

PSB inhibits tumor angiogenesis in the Calu-3 xenograft model. Angiogenesis performs an important role in the development and metastasis of cancers. To evaluate the potential anti-angiogenic mechanism of PSB *in vivo*, the lung adenocarcinoma Calu-3 cell tumors were analyzed by immunostaining for platelet endothelial cell adhesion molecule 1 (CD31). A significant dose-dependent reduction of CD31-positive endothelial cells was observed 3 h subsequent to the administration of 100 and 200 mg/kg PSB in Calu-3 cell tumors (Fig. 4A). At the 200 mg/kg PSB dose, PSB administration resulted in a 60-70% reduction in CD31-positive microvessels, which was significant compared with the control group (P<0.01) and indicated that the PSB-induced inhibition of Calu-3 tumor cell growth corresponds with the anti-angiogenic activity (Fig. 4B). However, the positive control of Herceptin only caused a mild, but not significant, reduction in CD31-positive microvessels.

Discussion

The importance of the phosphatidylinositol 3-kinase (PI3K)-Akt axis in oncogenic signaling is becoming increasingly apparent, particularly in the case of HER2-positive cancer (17). PI3K is responsible for diverse cellular regulation, including cell adhesion, motility, migration, proliferation, reduced apoptosis, anchorage independence and intracellular vesicle trafficking/secretion (18). Akt is the main downstream target of PI3K, and phosphorylation of Akt leads to enhanced cell survival (19). In *HER2*-amplified cancers, the heterodimer formed by HER2 and kinase-deficient HER3 is a major activator of PI3K-Akt signaling, and when phosphorylated, HER3 directly couples with the p85 subunit of PI3K (20). In the present study, PSB significantly inhibited the growth of *HER2*-amplified lung adenocarcinoma Calu-3 cell growth *in vitro* and *in vivo*. In addition, the results of western blot analysis revealed that p-HER2 and its downstream signaling molecules p-Akt and p-ERK were significantly inhibited by PSB. Therefore, the mechanism of PSB-induced inhibition of Calu-3 tumor cell growth may act through the regulation of the PI3K/Akt and ERK pathways. However, Akt and ERK are downstream signaling molecules not only for HER2, but also for other members of the epidermal growth factor receptor family. Out of these receptors, it is unclear if only HER2 phosphorylaztion was inhibited by PSB, and therefore additional investigation is required.

The proliferation of a network of blood vessels that penetrates into cancerous lesions, supplying nutrients and oxygen to the cells and removing waste products, is termed tumor angiogenesis. This process is initiated by cancerous tumor cells releasing molecules that send signals to the surrounding normal host tissue. This signaling activates certain genes in the host tissue that, in turn, produce proteins to encourage the growth of novel blood vessels (21). Wei *et al* (22) reported that *Scutellaria barbata* D. Don inhibited tumor angiogenesis

via suppression of the Hedgehog pathway in a colorectal cancer HT-29 cell xenograft model. Previous investigation has also revealed that PSB significantly inhibited the cell invasion and migration of human lung cancer 95-D cells in a concentration-dependent manner. The adhesion of 95-D cells to fibronectin was also inhibited by PSB. The expression of C-MET and E-cadherin was significantly down- and upregulated, respectively, in 95-D cells treated with PSB (23). In the present study, it was demonstrated that PSB inhibited tumor angiogenesis in the Calu-3 xenograft model via suppression of the HER2 signaling pathway. However, this result was inconsistent with the findings of Wei *et al* (22). It is possible that PSB exerts anti-angiogenesis effects in different cancer cell types through the inhibition of various signaling pathways.

In China, TCM has been used for >1,000 years to prevent and alleviate a wide variety of diseases. Certain agents used for the treatment of cancer have been derived from TCM (24,25). However, the exact antitumor growth mechanisms of TCM remain unclear, which hinders the usage of TCM in clinical cancer treatment. The present study aimed to explain the mechanism of antitumor effects of PSB in lung cancer cells. The results revealed that PSB not only downregulates the expression of p-HER2 in Calu-3 cells, but also blocks the downstream signaling pathways, including p-Akt and p-ERK. Immunohistochemical analysis in tumor sections revealed that the antitumor efficacy of PSB was mainly associated with anti-angiogenesis. The present study aids the understanding of the antitumor effect of PSB in lung cancer, which may support a breakthrough in the use of TCM for the radical treatment of malignancies.

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