Extracts from Huangqi (*Radix Astragali Mongolici*) and Ezhu (*Rhizoma Curcumae Phaeocaulis*) inhibit Lewis lung carcinoma cell growth in a xenograft mouse model by impairing mitogen-activated protein kinase signaling, vascular endothelial growth factor production, and angiogenesis

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**Abstract**

**OBJECTIVE:** To study the anti-tumor effects of the extracts from Huangqi (*Radix Astragali Mongolici*) and Ezhu (*Rhizoma Curcumae Phaeocaulis*) on the growth of Lewis lung carcinoma (LLC) in a xenograft model and to investigate the possible underlying mechanism.

**METHODS:** LLC tumor-bearing C57BL/6 mice were treated with normal saline, cisplatin (2 mg/kg intraperitoneally every other day), or Huangqi (*Radix Astragali Mongolici*) and Ezhu (*Rhizoma Curcumae Phaeocaulis*) (1:1, 2:1, or 3:1 ratio; 5, 8, or 11 g/kg crude drug intragastrically every day) for 15 d. Body weights and tumor volumes were measured every other day. Tumors were excised on day 15 and analyzed. Tumor microvessel density (MVD) was assessed by immunohistochemical staining of CD34; and expression of vascular endothelial cell growth factor (VEGF), the mitogen-activated protein kinases p38 mitogen-activated protein kinase (MAPK), extracellular signal-regulated kinases 1 and 2 (ERK1/2), and Jun N-terminal kinase (JNK) and their phosphorylated forms were assessed by Western blotting.

**RESULTS:** Treatment with cisplatin caused a significant loss of body weight compared with controls, whereas Huangqi (*Radix Astragali Mongolici*) and Ezhu (*Rhizoma Curcumae Phaeocaulis*) extract combinations had no effect. Extracts from Huangqi (*Radix Astragali Mongolici*) and Ezhu (*Rhizoma Curcumae Phaeocaulis*) significantly decreased tumor weight and tumor MVD compared with controls, and at the 3:1 treatment group had similar efficacy to cisplatin in reducing MVD. Tumors from Huangqi (*Radix Astragali Mongolici*) and Ezhu (*Rhizoma Curcumae Phaeocaulis*) treatments also showed decreased p38 MAPK, p-p38 MAPK, ERK1/2, p-ERK1/2, JNK, and p-JNK expression compared with the control group (all \( P < 0.01 \)). VEGF protein expression was significantly reduced in the 2:1 and 3:1 treatment groups compared with the control group (\( P < 0.01 \)).

**CONCLUSION:** Extracts from Huangqi (*Radix Astragali Mongolici*) and Ezhu (*Rhizoma Curcumae Phaeo-
caulis) hindered LLC growth in the xenograft mouse model, possibly via inhibition of the MAPK signaling pathway, VEGF production, and tumor angiogenesis.

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Keywords: Carcinoma, Lewis lung; Vascular endothelial cell growth factors; Extracellular signal-regulated MAP kinases; Angiogenesis; Huangqi (Radix Astragali Mongolic); Ezhu (Rhizoma Curcumae Phaeocaulis)

INTRODUCTION

Lung cancer is the leading cause of cancer death among all cancer types and comprises 26% of all cancer cases. Current first-line therapies for non-small cell lung cancer, which accounts for 80%-85% of all lung cancers, consist of a combination of chemotherapies and targeted therapies. Despite advances in many therapeutic areas, the 5-year survival rate for lung cancer is still less than 20%, indicating the need for additional therapies.

Cisplatin (DDP)-based chemotherapy has been widely used for the treatment of non-small cell lung cancer patients. However, its clinical use is limited by side effects, which include ototoxicity, nephrotoxicity, weight loss, and gastrointestinal disturbances such as vomiting. In recent years, there has been increasing interest in the use of plant-derived products, including Chinese herbal medicines, for cancer therapy. Many naturally occurring plant-derived products, such as epipodophyllotoxin, vincristine, camptothecin, and paclitaxel, have been demonstrated as valuable cancer therapies. As a complementary and alternative medicine commonly used in China, Traditional Chinese Medicine (TCM) has some advantages for the treatment and prevention of various cancers due to the low cost and ability to improve quality of life. As a result, research of Chinese medicines as anti-tumor agents is rapidly increasing. Huangqi (Radix Astragali Mongolic) and Ezhu (Rhizoma Curcumae Phaeocaulis) are commonly used in TCM to treat a variety of cancers, and many active ingredients of them have been reported to show anti-tumor effects.

Angiogenesis plays an important role in tumor growth and metastasis. As a pathological process, angiogenesis can promote tumor progression by supplying sufficient oxygen and nutrients. Within the solid tumor, angiogenesis is regulatory by two sets of molecules with opposing functions; perturbing the balance of angiogenic and anti-angiogenic factors can make the tumor develop and metastatic. The mitogen-activated protein kinase (MAPK) signaling pathway is a critical regulator of tumor angiogenesis, several studies have demonstrated ed roles for the MAPK signaling pathway in angiogenesis.

In this study, we examined the effects of extracts from Huangqi (Radix Astragali Mongolic) and Ezhu (Rhizoma Curcumae Phaeocaulis) on the growth of Lewis lung carcinoma (LLC) in a xenograft mouse model and investigated the possible mechanism.

METHODS

Mice, cells, and reagents

Specific pathogen-free male C57BL/6 mice (6-8 weeks of age) were purchased from Beijing Vital River Laboratory Animal Technology Co. Ltd. China (certificate No. SCXK [Jing] 2016-0011). Mice were housed under standard conditions in pathogen-free rooms on a 12-h light-dark cycle and were supplied with food and water ad libitum. All experiments were performed following the guidelines of the China Institute of Laboratory Animal Science.

LCC cells were purchased from the Cell Center of the Chinese Academy of Medical Sciences (Beijing, China). Antibodies were obtained as follows: anti-CD34 and anti-VEGF were from Abcam (Cambridge, UK); antibodies to ERK1/2, JNK, p38 MAPK, phospho (p)-ERK1/2, p-JNK, p-p38 MAPK, and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) were from Cell Signaling Technology (Danvers, MA, USA); and anti-β-actin, goat anti-rabbit IgG-horseradish peroxidase (HRP), and goat anti-mouse IgG-HRP were from CWbio Co. (Beijing, China). cis-diaminedichloroplatinum II (cisplatin, DDP) for injection was obtained from Hospira (Mulgrave, Australia).

Crude extracts from Huangqi (Radix Astragali Mongolic) and Ezhu (Rhizoma Curcumae Phaeocaulis) were purchased from the Chinese PLA General Hospital (Beijing, China). We prepared three experimental mixtures of Huangqi (Radix Astragali Mongolic) and Ezhu (Rhizoma Curcumae Phaeocaulis) (1 : 1, 2 : 1, 3 : 1) in distilled water equivalent to eight times the herb weight and decocted twice for 40 min. The decoctions were collected, combined, and concentrated to the corresponding dose. The decoctions were stored at 4 °C prior to intragastric (i.g.) administration as described below. Doses for the mouse treatments were based on the clinical dose for a 70 kg adult human and were extrapolated from the weight of the starting crude mixture of Huangqi (Radix Astragali Mongolic) and Ezhu (Rhizoma Curcumae Phaeocaulis). The three doses used were 5, 8, or 11 g crude drug equivalent per kg body weight, which corresponded to 1 : 1, 2 : 1, and 3 : 1 mixtures of Huangqi (Radix Astragali Mongolic) and Ezhu (Rhizoma Curcumae Phaeocaulis), respectively.
Mouse experiments
Cultured LLC cells were harvested, washed twice with ice-cold phosphate-buffered saline (PBS), and resuspended at 1 × 10^6 live cells (by trypan blue staining) per mL PBS. Aliquots of 0.1 mL cell suspension were injected subcutaneously into the left armpits of the mice. The next day, the mice were assigned to five groups in a randomized block design: (a) the Model group (n = 9) was administered 0.9% normal saline (NS, 0.1 mL/10 g body weight) i.g. daily on days 1-15 and injected intraperitoneally (i.p.) with NS (0.1 mL/10 g) on days 1, 3, 5, 7, 9, 11, 13, and 15; (b) the DDP group (n = 9) was administered NS as above and injected i.p. with DDP 2 mg/kg on days 1, 3, 5, 7, 9, 11, 13, and 15; (c) the 1:1 group (n = 8) was administered the 1:1 decoction (5 g crude herb equivalent/kg) i.g. daily on days 1-15 and injected i.p. with NS on days 1, 3, 5, 7, 9, 11, 13, and 15; and (d) and (e) the 1:2 and 1:3 groups (n = 8) were treated as described for (c), except the final doses of Huangqi (Radix Astragali Mongolici) and Ezhu (Rhizoma Curcumae Phaeocaulis) decoction were 8 g (d) and 11 g (e) crude herb equivalent/kg body weight.

Tumor length and width were measured with Vernier calipers every 2 d, and the volumes were calculated as: 0.5 × tumor width^2 × tumor length. 37 Body weights were measured with an electronic balance every 2 d. On day 16, the mice were sacrificed and the tumors were harvested and weighed. The percentage of tumor inhibition was calculated as: (1-average weight of tumors in treatment group/average weight of tumors in Model group) × 100%. 38 Portions of the tumor specimens were fixed in 4% paraformaldehyde for the IHC study, and the remaining tissues were kept at -80 °C for Western blot analysis.

Immunohistochemistry
Fixed tumor samples (eight per group) were embedded with paraffin, sectioned (5 μm thick), and dewaxed according to standard procedures. Antigen retrieval was performed by microwaving the slides, and endogenous peroxidase activity was quenched by incubating with 3% hydrogen peroxide. The sections were then incubated with anti-CD34 antibody (at 1:500 dilution) overnight at 4 °C, washed and then incubated with IgG-HRP-conjugated secondary antibody for 30 min at 37 °C. Color was developed using a 3, 3’-diaminobenzidine (DAB) kit (Cervicebio, Wuhan, China). Images were captured using an Olympus BX60 microscope. Microvessel density (MVD) was calculated as the number of positively stained cells in four high-power fields per tumor section. 39

Western blot analysis
Tumor issues (100 mg) were cut into small pieces on ice, washed twice with cold PBS, and then homogenized using a glass tissue grinder in 1 mL RIPA lysis buffer containing protease and phosphatase inhibitors. The homogenized samples were incubated on ice for 10 min and centrifuged at 10 000 × g for 10 min at 4 °C . Supernatants were collected and protein was quantified using a BCA assay (CWbio Co.). Samples were mixed with loading buffer, heated at 100 °C for 10 min, and resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Proteins were then electro-transferred onto polyvinylidene difluoride membranes (0.45 μm pore size HATF; Millipore, Burlington, MA, USA) and the membranes were blocked with 5% bovine serum albumin for 2 h at room temperature. The membranes were then incubated with the appropriate primary antibodies overnight at 4 °C (all at 1:1000 dilution), washed five times with Tris-buffered saline containing 0.05% Tween 20 (TBST) for 5 min each, and then incubated with the appropriate HRP-conjugated secondary antibodies (goat-anti-mouse or goat-anti-rabbit IgG, 1:3000 dilution) for 1 h at room temperature. Finally, blots were washed five times with TBST, and antibody binding was detected using a high-sensitivity chemiluminescence detection kit (CWbio Co.). Images were captured using an Image Quant LAS 500 system (Healthcare Bio-Sciences AB, Uppsala, Sweden).

Statistical analysis
All data are expressed as the mean ± standard deviation (± s ± i) of the indicated number of replicates. Data were analyzed using SPSS 17.0 (SPSS Inc. Released 2008, SPSS Statistics for Windows, Version 17.0, Chicago, IL, USA). One-way analysis of variance was performed followed by the Student-Newman-Keuls post hoc test to test the differences between groups. A P value < 0.05 was considered statistically significant.

RESULTS
Effect of Huangqi (Radix Astragali Mongolici) and Ezhu (Rhizoma Curcumae Phaeocaulis) extract combinations on body weight and LLC tumor growth in the model
Since Chinese medicines can be used in various combinations with great flexibility, it is important to determine accurate dose-response relationships for these medicines when used individually and in combination. 40 We examined the anti-tumor effects of various proportions of Huangqi (Radix Astragali Mongolici) and Ezhu (Rhizoma Curcumae Phaeocaulis) in combination on a mouse xenograft model established with LLC cells and compared the efficacy with that of DDP. Mice were injected with LLC cells and treated with Huangqi (Radix Astragali Mongolici) and Ezhu (Rhizoma Curcumae Phaeocaulis) or DDP. On day 16, the mice were sacrificed and the tumors were removed and photographed (Figure 1). There were no significant inter-group differences in mouse body weight at the start of the experiment. However, the DDP group showed
slower weight gain during the experiment compared with the Model or herbal medicine-treated groups, and a decrease in weight in DPP-treated mice compared with other groups was evident by day 9 (Figure 2B). The tumor volumes did not differ significantly between groups during the initial days of treatment intervention, but the tumors in the DDP and herbal medicine groups grew more slowly compared with the Model group at later times. Tumor growth was most effectively inhibited by DDP, followed by 3∶1, 2∶1, and 1∶1 treatments (Figure 2A).

Figure 1 Images of representative tumors from each mouse group

Tumors from the DDP- and Huangqi (Radix Astragali Mongolici) / Ezhu (Rhizoma Curcumae Phaeocaulis)-treated groups weighed significantly less than the tumors from the Model control group (P < 0.01, Figure 2C), and tumors from the DDP-treated group weighed significantly less than those from the herbal medicine-treated groups (P < 0.05). In comparing the herbal medicine-treated groups, the 3∶1 and 2∶1 combinations were more effective than the 1∶1 combination in inhibiting tumor growth, but there was no significant difference between the efficacy of the 3∶1 and 2∶1 combinations (Figure 2C). The percentage of tumor inhibition for the DDP; 3∶1, 2∶1, and 1∶1 groups was 61.69%, 43.21%, 37.70%, and 24.89%, respectively (Figure 2D).

Effect of the Huangqi (Radix Astragali Mongolici) and Ezhu (Rhizoma Curcumae Phaeocaulis) combinations on tumor MVD

As shown in Figure 3A, CD34 expression was detected as yellow-brown staining in the cytoplasm and membranes of vascular endothelial cells, with a diffuse distribution of most of microvascular vessels evident at the edge of the tumors. Notably, the MVD of tumors from DDP- and Huangqi (Radix Astragali Mongolici)/Ezhu(Rhizoma Curcumae Phaeocaulis)-treated groups was significantly lower than that of the Model group (P < 0.01). Although DDP had a greater effect on reducing MVD than all the three herbal medicine combinations (P < 0.05), the effect of the 3∶1 combination was most similar to DDP (Figure 3B).

Figure 2 Effect of Huangqi (Radix Astragali Mongolici) and Ezhu (Rhizoma Curcumae Phaeocaulis) combination treatment on mouse body weights and LLC tumor growth

Change in mean body weight (A) and tumor volume (B). The tumor weight (C) and tumor inhibitory rate (D) of the five groups. Model group (n = 9) was administered NS i.g. daily on days 1-15 and injected i.p. with NS on days 1, 3, 5, 7, 9, 11, 13, and 15; DDP group (n = 9) was administered NS as above and injected i.p. with DDP 2 mg/kg on days 1, 3, 5, 7, 9, 11, 13, and 15; 1∶1; 2∶1 and 3∶1 group (n = 8) were administered the corresponding dose decoction i.g. daily on days 1-15 and injected i.p. with NS on days 1, 3, 5, 7, 9, 11, 13, and 15. DDP: cisplatin; 1∶1; 2∶1 and 3∶1 group: the three doses were 5, 8 and 11 g crude Huangqi (Radix Astragali Mongolici) and Ezhu (Rhizoma Curcumae Phaeocaulis) equivalent/kg body weight. *P < 0.01 compared with the Model group; †P < 0.05 compared with the DDP group.
Effect of Huangqi (Radix Astragali Mongolici) and Ezhu (Rhizoma Curcumae Phaeocaulis) combinations on expression of MAPK signaling pathway components and VEGF

We next evaluated MAPK signaling pathways and VEGF in tumors from the treatment groups (Figure 4A). p38 MAPK and p-p38 MAPK protein expressions were not significantly different in tumors from the Model and DDP-treated groups (Figure 4B, C). In contrast, both protein levels were significantly lower in the Huangqi (Radix Astragali Mongolici)/Ezhu (Rhizoma Curcumae Phaeocaulis)-treated groups than in the Model group (P < 0.01) and also lower in all three herbal medicine-treated groups compared with the DDP-treated group (P < 0.01); however, there were no significant differences between the Huangqi (Radix Astragali Mongolici) and Ezhu (Rhizoma Curcumae Phaeocaulis) groups. Expressions of ERK1/2 and p-ERK1/2 were significantly lower in tumors from the DDP- and herbal medicine-treated groups compared with the Model group (P < 0.01) and significantly lower in the 2:1-treated groups compared with the DDP-treated group (P < 0.01) (Figure 4D, E). All three herbal medicine-treated groups showed significantly reduced JNK and p-JNK protein expression compared with the Model group (P < 0.01) (Figure 4F, G); however, only p-JNK protein expression in the 2:1-treated group was significantly lower compared with the DDP group (P < 0.01). VEGF protein expression was significantly lower in tumors from the DDP-, 2:1-, and 3:1-treated groups than the Model group (P < 0.01) and lower in the 3:1 group than in the DDP group (P < 0.01) (Figure 4H, I).

DISCUSSION

In the study we found that the extracts from Huangqi (Radix Astragali Mongolici) and Ezhu (Rhizoma Curcumae Phaeocaulis) can inhibit LLC growth in the xenograft mouse model without impacting mouse body weight. Moreover, tumors from the untreated control mice exhibited a steep growth curve, DDP and all three herbal medicine combinations were able to slow tumor growth, with DDP being the most effective. DDP and the Huangqi (Radix Astragali Mongolici) and Ezhu (Rhizoma Curcumae Phaeocaulis) combinations also significantly reduced tumor weights measured at the end of the experiment, with the 3:1 combination group having similar effects to DDP group. Maintenance of body weight during cancer treatment is an important contributor to patient quality of life. Thus, although the growth inhibitory effects of the extracts were weaker than that of DDP, the maintenance of body weight during treatment represents a distinct advantage, especially since improvements in quality of life are basic tenets of the holistic concepts of TCM. Tumor angiogenesis plays an important role in tumor growth and metastasis. Among the active ingredients of Huangqi (Radix Astragali Mongolici) and Ezhu (Rhizoma Curcumae Phaeocaulis), Astragalus saponins, Astragalus polysaccharides, β-elemene, and curcumin have been reported to exhibit anti-angiogenic effects. 21–23 In this study, we analyzed the expression of the CD34 in vascular endothelial cells as a marker of tumor MVD and angiogenesis. CD34 staining enables visualization not only of the small immature microvessels but also of single vascular endothelial cells in the tumor. We found that the Huangqi (Radix Astragali Mongolici) and Ezhu (Rhizoma Curcumae Phaeocaulis) treatments significantly reduced MVD compared with the control group. VEGF is an important regulator of angiogenesis, and VEGF-specific monoclonal antibodies and small molecule inhibitors can block the growth of several human tumor cell lines in nude mice. 24 We found that VEGF protein expression was signifi-
The MAPK signaling pathway is a critical regulator of tumor angiogenesis. Several studies have shown that ERK1/2 activation increases the transcription of VEGF, and JNK activation can improve the stability of VEGF mRNA. In squamous cell carcinoma of the head and neck, p38 MAPK signaling regulates cancer cell growth and tumor angiogenesis by inhibiting the VEGF activation. Therefore, targeting key components in the MAPK signaling pathway may be one mechanism to inhibit tumor angiogenesis. Osumi’s research found that selumetinib (MEK1/2 inhibitor) alone or with cediranib reduced the ERK phosphorylation, angiogenesis, and tumor cell proliferation and increased apoptosis of the tumor cell. Our study shows that the extracts from Huangqi (Radix Astragali Mongolici) and Ezhu (Rhizoma Curcumae Phaeocaulis) reduces the effects of extracts from the combinations.
pression of ERK1/2, p38 MAPK, and JNK and their active phosphorylated forms in LLC tumor xenografts. Moreover, the different ratios of Huangqì (Radix Astragali Mongolici) and Ezhu (Rhizoma Curcumae Phaeo-)

caulis) have different degrees of down-regulation of the above-mentioned key factors, suggesting that there may be some differences in the mechanism of action.

In conclusion, our results indicate that the extracts from Huangqì (Radix Astragali Mongolici) and Ezhu (Rhizoma Curcumae Phaeo-caulis) inhibit LLC growth in a xenograft mouse model without affecting its body weight. The mechanism behind the action may involve the downregulation of active p38 MAPK, ERK1/2, and JNK and inhibition of the downstream target VEGF.

REFERENCES


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