Traditional Chinese Medicine Brucea Javanica Oil Enhances the Efficacy of Anlotinib in a Mouse Model of Liver-Metastasis of Small-cell Lung Cancer

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Abstract. *Background/Aim:* Small-cell lung cancer (SCLC) is a recalcitrant disease with liver and other metastasis. The present study evaluated the efficacy of the traditional Chinese medicine Brucea javanica oil (BJO) combined with anlotinib, a multi-tyrosine kinase inhibitor with anti-angiogenic activity, on a nude-mouse model of SCLC liver metastasis. *Materials and Methods:* The mouse model was established by injecting NCI-H446 cells (1×10^6) in Matrigel (20 μ l) into the upper liver lobe. All animals were randomized and assigned to three groups: Control (n=8); anlotinib alone (n=8; 3 mg/kg, qd $\times$ 14+7-day interval with two cycles, oral); anlotinib plus BJO (n=8; 3 mg/kg anlotinib qd $\times$ 14+7-day interval with two cycles, orally; BJO: 1 g/kg, qd $\times$ 6 weeks, orally). Body weight was determined every week. Six weeks after initial treatment, tumors were collected for analysis of angiogenesis using immunohistochemistry. *Results:* The combination of anlotinib and BJO significantly inhibited growth of SCLC liver metastases and angiogenesis more than anlotinib monotherapy ($p=0.043$). In addition, BJO alleviated body-weight loss associated with anlotinib therapy, including general mouse condition. *Conclusion:* The results of the present study indicate that the combination of anlotinib with

BJO is promisingly active against liver metastases of SCLC, and has clinical potential.

Lung cancer is responsible for the greatest number of cancer deaths in the world (1, 2). There are two major types of lung cancer according to histopathological classification: non-small-cell lung cancer and small-cell lung cancer (SCLC). SCLC is an aggressive neuroendocrine malignancy with a high proliferative index, which accounts for 15% of all lung cancer (3, 4). However, because of its rapid progression and early metastasis (5), the 5-year survival rate of patients with SCLC is less than 5%, lower than that for non-small-cell lung cancer (6). Although cytotoxic therapy has initial efficacy for SCLC, there is recurrence in most patients (7). Recurrent metastasis is recalcitrant, with only 5% survival (8).

Brucea javanica, a traditional Chinese medicine listed in the official Chinese Pharmacopoeia (9), is an evergreen plant shrub from the Simaroubaceae family that grows abundantly throughout Southern China and Southeast Asia (10). Oil from *B. javanica* (BJO) has been used to treat diarrhea (11), malaria (12), intestinal inflammation (13), and various types of cancer (9, 14-17).

Anlotinib is a small-molecule, multi-targeting tyrosine kinase inhibitor, which can inhibit vascular endothelial growth factor receptors 2, and 3, platelet-derived growth factor receptor, and stem cell-factor receptor (18). Based on the ALTER 1202 clinical trial, the China Food and Drug Administration approved anlotinib for use in third-line treatment in recurrent SCLC or SCLC with distant metastasis (19).

We previously reported that BJO enhanced gemcitabine efficacy in a mouse model of pancreatic cancer (20). In the present study, we combined BJO with anlotinib for the treatment of SCLC liver metastasis in a nude-mouse model.

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Key Words: *Brucea javanica* oil, small-cell lung cancer, SCLC, nude mouse, liver metastasis, anlotinib, angiogenesis, combination therapy, efficacy.

Materials and Methods

Cell culture. The human SCLC cell line NCI-H446 was purchased from the American Type Culture Collection (Manassas, VA, USA). Cells were maintained in RPMI-1640 culture medium supplemented with 10% heat-inactivated fetal bovine serum and 1% penicillin, and cultured at 37°C in an incubator with 5% CO₂.

Animals. A total of 24 female athymic nude (nu/nu) mice, purchased from Cavens Inc. (Changzhou, Jiangsu, China), 5-6 weeks old, 20±2 g body weight, were used for the study. All animals were maintained in a high-efficiency particulate air-filtered environment at the animal research laboratory of Anhui medical university (Hefei, Anhui, PR China). Cages, food and bedding were autoclaved.

Experimental agents. Anlotinib was purchased from Chia Tai Tianqing Pharmaceutical Group Co., Ltd (Lian Yungang, Jiangsu, PR China). Anlotinib was dissolved in dimethyl sulfoxide to prepare stock solution at a concentration of 16 mmol/l, and stored at -20°C. Before treatment, the stock solution was diluted to 0.75 mg/ml using distilled water.

BJO capsules were purchased from Vanguard Pharmaceutical (Haimen, Jiangsu, PR China). BJO was dissolved in distilled water to a concentration of 250 mg/ml, and stored at 4°C.

Establishment of a nude mouse model of liver metastasis from SCLC. An SCLC liver-metastasis mouse model was established by injecting NCI-H446 cells (1×10⁶) in Matrigel (20 µl) (BD, Franklin Lakes, NJ, USA) into the upper liver lobe of nude mice. All surgical procedures were performed under 8× magnification in a high-efficiency particulate air-filtered laminar flow hood. Animals were anesthetized by intramuscular injection of a ketamine mixture. The surgical area was sterilized using iodine and alcohol. A transverse incision of approximately 1 cm was made on the upper abdomen of the mouse using surgical scissors. The liver was exposed, and NCI-H446 cells (20 µl) were injected into the liver lobe. Bleeding at the injection site was stopped by cotton swab pressure. The incision was closed with 5-0 surgical sutures.

Treatment. Treatment was started 1 week after NCI-H446-cell injection. All animals were randomized and assigned to three groups of eight animals each: Control; anlotinib (3 mg/kg, qd×14+7-day interval for two cycles, oral administration by gavage) (21); anlotinib as above plus BJO (1 g/kg, qd×6 weeks, gavage) (20). Body weight was determined every week using an electrical scale throughout the treatment period. The study was terminated 6 weeks after the initiation of treatment. All animals were euthanized and examined at necropsy. The abdominal cavity was opened to expose the liver. Tumor volume was estimated by measuring the perpendicular minor dimension (W) and major dimension (L). Approximate tumor volume was calculated with the formula (W²×L)/2. The tumors were resected and tumor weight was determined using an electronic scale. The tumor growth inhibition (TGI) in each group was calculated with the formula: TGI (%)=(1–mean tumor weight of the treated group/mean tumor weight of the control group) ×100%. The tumor samples were stored in 10% formalin for histopathological examination.

Immunohistochemistry. The expression of CD34 was detected with an immunohistochemical assay to compare the extent of tumor

microvessel area between each group. Formaldehyde-fixed tumors were embedded in paraffin and the sections (4 µm) were obtained using a microtome (RM2125; Leica, Wetzlar, Germany). Sections were deparaffinized after hydration, and antigen retrieval buffer was used for 30 minutes. After endogenous peroxidase was quenched with 0.1% hydrogen peroxide for 15 minutes, the samples were blocked using serum-free protein and incubated with primary rabbit monoclonal against CD34 (ab110643; Abcam, Cambridge, MA, USA) overnight. Following washing with phosphate buffer saline, sections were incubated with a sheep anti-rabbit IgG secondary antibody (ab172730; Abcam), followed by incubation with 3% peroxidase (Invitrogen, Waltham, MA, USA) and a solution of 0.1% 3, 3-diaminobenzidine and 0.02% H₂O₂. The sections were examined and positive cells were recognized by their brown-stained appearance. Expression levels were quantified by the average optical density of the positive cells in five fields/sample with Image-Pro Plus 6.0 software (Media Cybernetics, Silver Springs, MD, USA) (22). Typical images were captured using a microscope (BX53F; Olympus Corp, Tokyo, Japan) with its software.

Statistical analysis. All data were analyzed with GraphPad Prism 8.2.1 software (GraphPad Software Inc., La Jolla, CA, USA). All data are represented as the mean±standard deviation. One-way analysis of variance followed by Tukey's correction were used to determine statistical differences in the mean tumor volume, tumor weight and body weight among the experimental groups. Values of $p \leq 0.05$ were considered statistically significant.

Results

BJO enhances anlotinib efficacy against liver metastasis from SCLC. All treatment groups resulted in statistically significantly smaller tumor volumes (Figure 1) and weights compared to the vehicle-treated control at the end of treatment ($p < 0.05$). The addition of BJO significantly increased efficacy compared to the anlotinib monotherapy, both for tumor volume ($p = 0.024$) and tumor weight ($p = 0.037$) (Table I). The TGI value in the combination-treated group was 53.44% compared to that of anlotinib monotherapy of 21.82%.

BJO reduced anlotinib-induced weight loss in the nude-mouse model of SCLC liver metastasis. Mice treated with the combination of anlotinib and BJO demonstrated significantly lower body-weight loss compared to those on anlotinib monotherapy during the entire treatment period ($p = 0.043$) (Figure 2). The greater activity and smoother skin of the combination-treated mice indicated that BJO improved their general condition.

BJO enhanced the anti-angiogenic effect of anlotinib. Although anlotinib monotherapy significantly inhibited growth of tumor microvessels compared to the vehicle control group ($p = 0.034$), the combination of anlotinib and BJO showed significantly higher anti-angiogenic efficacy compared to mice of vehicle control ($p = 0.001$) and anlotinib monotherapy ($p = 0.023$) group (Figure 3).

Figure 1. Inhibitory effect of the combination of *Brucea javanica* oil and anlotinib on small cell lung cancer liver metastases in a nude-mouse model. Images of liver metastasis tumor in each group at necropsy, liver metastases are shown with yellow arrows and dashed line.

Table I. Efficacy of treatment on final tumor volume and weight in the nude-mouse model of liver metastasis of small-cell lung cancer.

Group	Tumor volume (mm ³) Mean±SD	p-Value	Tumor weight (g) Mean±SD	p-Value
Control	396.9±70.8		2.87±0.65	
Anlotinib	302.5±57.5	0.025*	2.24±0.44	0.231*
Anlotinib+BJO	180.8±48.8	0.001* 0.024[#]	1.34±0.45	0.008* 0.037[#]

Bold values indicate statistically significant difference. *Versus* *Control, [#]anlotinib-treated.

Discussion

The results of the present study indicate that the traditional Chinese medicine BJO both enhanced the efficacy of anlotinib on the growth of the SCLC liver metastases and prevented toxic body weight loss due therapy with anlotinib. SCLC is a recalcitrant disease in need of effective therapy (23). Although first-line chemotherapy is often effective for SCLC, the effect is temporary and there is almost always recurrence which leads to the patient's death. Liver

Figure 2. Effect of *Brucea javanica* oil on mouse body weight during the treatment period. Data are presented as the mean±standard deviation. *Significantly different from anlotinib monotherapy at $p<0.05$.

metastasis, along with brain metastasis, are the major causes of death in patients with SCLC (24, 25). Although anlotinib has been approved for third-line therapy, in China it has shown only moderate clinical efficacy (18), and also showed moderate efficacy in the present study. In addition, drug-related adverse reactions are often found in patients, including fatigue (17.7%), weight loss (17.7%), and hematological toxicity (13.9%) (26). The dose of anlotinib used in this study had significant toxicity, as shown by mouse body-weight loss.

Our previous study showed that BJO enhanced gemcitabine efficacy and increased survival in a patient-derived orthotopic xenograft mouse model of pancreatic cancer (20). In the present study, we first showed BJO can be effectively combined with anlotinib in a nude-mouse model of SCLC liver-metastasis, where in combination with anlotinib it inhibited both liver metastasis growth and tumor angiogenesis in the liver. BJO also alleviated the weight loss due to anlotinib, and improved mouse body condition.

In brief, BJO both enhanced anlotinib efficacy while reducing toxicity, indicating the future clinical potential of the combination of BJO and anlotinib for SCLC liver-metastasis, a recurrently recalcitrant disease.

Conflicts of Interest

All the Authors declare no conflicts of interest in this study.

Figure 3. Anti-angiogenic efficacy of the combination of *Brucea javanica* oil and anlotinib. Representative images are shown of CD34 (brown)-stained tumor reflecting angiogenesis in each group (200 $\times$ magnification). A: Control; B: anlotinib monotherapy; C: BJO combined with anlotinib. D: Quantification of tumor microvessel area was quantified by the average optical density (AOD) in each group. Data are presented as the mean $\pm$ standard deviation. Significantly different at * p <0.05 and *** p <0.001.

Authors' Contributions

SP designed the study. SP, WD, QC, JM performed the experiments. YS analyzed the data. SP and HY drafted the article. RMH revised the article and rewrote the Discussion. JH administrated and supervised the study.

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