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1-o-acetylbritannilactone (ABL) inhibits angiogenesis and lung cancer cell growth through regulating VEGF-Src-FAK signaling

He Zhengfu [a], Zhang Hu [a], Miao Huiwen [a], Li Zhijun [a], Zhou Jiaojie [b], Yan Xiaoyi [b, *], Cai Xiujun [c, **]

[a] Department of Thoracic Surgery, Sir Run Run Shaw Hospital of Zhejiang University School of Medicine, Hangzhou, China
[b] Zhejiang University School of Medicine, Hangzhou, China
[c] Sir Run Run Shaw Hospital of Zhejiang University School of Medicine, Hangzhou, China

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ABSTRACT

The search for safe, effective and affordable therapeutics against non-small cell lung cancer (NSCLC) and other lung cancers is important. Here we explored the potential effect of 1-o-acetylbritannilactone (ABL), a novel extract from Inula britannica-F, on angiogenesis and lung cancer cell growth. We demonstrated that ABL dose-dependently inhibited vascular endothelial growth factor (VEGF)-induced proliferation, migration, and capillary structure formation of cultured human umbilical vascular endothelial cells (HUVECs). *In vivo*, ABL administration suppressed VEGF-induced new vasculature formation in Matrigel plugs. For the mechanism investigations, we found that ABL largely inhibited VEGF-mediated activation of Src kinase and focal adhesion kinase (FAK) in HUVECs. Furthermore, treatment of A549 NSCLC cells with ABL resulted in cell growth inhibition and Src-FAK in-activation. Significantly, administration of a single dose of ABL (12 mg/kg/day) remarkably suppressed growth of A549 xenografts in nude mice. *In vivo* microvessels formation and Src activation were also significantly inhibited in ABL-treated xenograft tumors. Taken together, our findings suggest that ABL suppresses angiogenesis and lung cancer cell growth possibly via regulating the VEGFR-Src-FAK signaling.

1. Introduction

Non-small cell lung cancers (NSCLCs) and other forms of lung cancers cause significant mortalities worldwide annually [1–3]. Surgery resection is only curable for the patients with early stage diseases and decent conditions [1–3]. Due to lack of characteristic clinical manifestations, the fast majority of lung cancer patients are already with advanced/metastatic malignancies at the time of diagnosis [1–3]. Meanwhile, lung cancer cells often have profound pre-existing or acquired chemo-resistance to many known chemo-agents [1–3]. Thus, scientists are searching for safe, effective and affordable new therapeutics against this disease [1–3].

1-o-acetylbritannilactone (ABL) is a novel active compound isolated from Inula Britannica L [4]. The latter is one of the most popular and multi-purpose traditional Chinese medicinal herbs [4]. ABL is a 1,10-seco-eudesmanolide sesquiterpene extract from I. Britannica [4]. Existing evidences have shown that ABL could exert several biological functions including anti-inflammatory, anti-bacterial, anti-hepatitis, anti-diabetes, and anti-tumor activities [5–10]. ABL demonstrates chemo-preventive properties by inducing cell apoptosis in breast and ovarian cancers [5–10]. However, the activity and the molecular targets of ABL in lung cancers have not been determined.

Angiogenesis plays a vital role in cancer metastasis and patients' mortality [11,12]. Tumor vascularity, the feature of angiogenesis, has been considered as a prognostic factor in lung cancer and other solid tumors [11,12]. The glycoprotein vascular endothelial growth factor (VEGF) is widely expressed in many cancers, and is a critical component of tumor angiogenesis [11,12]. VEGF signaling regulating angiogenesis is mainly mediated by VEGF receptor 2 (VEGFR2), and activation of its downstream signaling targets, including Src kinase-focal adhesion kinase (FAK) pathway and others. These signalings work together or separately to promote endothelial cell survival, growth, migration and capillary tube formation [11,12]. Although VEGF signaling inhibitors (i.e. bevacizumab) have been approved for cancer treatment, their cost, toxicity and often lack of effectiveness are major roadblocks [11,12].

* Corresponding author. Zhejiang University School of Medicine, Hangzhou, China.
** Corresponding author. Sir Run Run Shaw Hospital of Zhejiang University School of Medicine, Hangzhou, China.
E-mail addresses: xiaoyiyan163@163.com (Y. Xiaoyi), xiujuncaomaj@163.com (C. Xiujun).

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In the current study, we show that ABL inhibits angiogenesis and lung cancer cell growth probably via suppressing VEGFR-downstream Src-FAK signaling.

2. Material and methods

2.1. Chemicals and reagents

ABL was synthesized and provided by Suzhou Ming-de Biotech (Suzhou, China) based on the protocol of [4]. VEGF was purchased from Sigma (Shanghai, China). Goat anti-rabbit or mouse horseradish peroxidase (HRP)-conjugated IgGs were purchased from Santa Cruz biotechnology (Santa Cruz, CA). All other antibodies utilized in this study were purchased from Cell Signaling Tech (Beverly, MA).

2.2. Cell culture

Human umbilical vein endothelial cells (HUVECs) were isolated from human umbilical cord veins by collagenase treatment. The harvested cells were grown in medium 199 (Gibco, Shanghai, China) containing 20% heat-inactivated fetal calf serum (FCS, Gibco), endothelial cell growth supplement (ECGS, 30 μg/mL, Sigma), epidermal growth factor (EGF 10 ng/mL, Sigma), 100 U/mL penicillin, and 100 μg/mL streptomycin. After 3–5 passages, HUVECs were collected for use in all experiments. All clinical investigations were approved by the institutional review board of authors' institutions, and were conducted according with the principles expressed in the Declaration of Helsinki. A549 human NSCLC cell line was purchased from Cell Bank of Chinese Academy of Medical Sciences (Shanghai, China). A549 cells were cultured in DMEM medium supplemented with 10% fetal bovine serum (FBS, Gibco) with antibiotics.

2.3. MTT assay of cell growth

The cell growth was measured by the 3-[4,5-dimethylthylthiazol-2-yl]-2,5 diphenyltetrazolium bromide (MTT) method. Briefly, cells were seeded in 96-well plates at a density of 4×10^3 cells/well. After treatment, MTT tetrazolium (5 mg/mL, Sigma) was added for 2 h. The medium was then aspirated, and 150 μL/well of DMSO was added. The absorbance was obtained using a plate reader at a test wavelength of 490 nm. The optical density (OD) value of treatment group was normalized to that of control group.

2.4. Clonogenicity assay

HUVECs or A549 cells were plated in 60 mm plates (300 cells/plate). After overnight incubation, cells were treated with applied agents for 24 h. Cells were then washed, and fresh media were added. After 10 days of incubation, surviving colonies were fixed, stained, and manually counted.

2.5. "Scratch" assay

Twelve-well plates were pre-coated with poly-lysine (30 μg/mL, Sigma), followed by further bovine serum albumin (BSA) blocking. A sufficient number of cells were plated, so that they became confluent in the wells right after attachment. Same area of each well was then displaced by scratching a same straight line through the layer with a needle. Floating cells were washed away by warm PBS. Cells were further incubated with VEGF (25 ng/mL), or with indicated concentration of ABL for 24 h. Mitomycin C (1 μg/mL, Sigma) was always included in the culture media to prevent cell proliferation.

2.6. Tube formation assay

Various concentration of ABL was added to HUVECs for 30 min before seeding and plated onto the layer of Matrigel at a density of 1×10^4 cells/well, and followed by the addition of 25 ng/mL VEGF. After 24 h, the enclosed networks of complete tubes from five randomly chosen fields were counted and photographed under a microscope. The number of formed capillary tubes were manually counted. Mitomycin C (1 μg/mL, Sigma) was added to prevent cell proliferation.

2.7. In vivo Matrigel plug assay

Briefly, Matrigel (9 mg/mL; 0.3 mL/mouse) containing 100 ng of VEGF, 20 units of heparin and/or 20 μmol of ABL were *s.c.* injected into the ventral area of 6-week-old C57BL/6 mice. Ten mice were used for each group. After 6 days, the skin of each mouse was pulled back to expose an intact Matrigel plug. IHC staining was performed to identify the formation and infiltration of new, functional microvessels.

2.8. Western-blots

Cells were harvested by HEPES lysis buffer (30 mM HEPES, 1% Triton X-100, 10% glycerol, 5 mM $MgCl_2$, 25 mM NaF, 1 mM EDTA and 10 mM NaCl). Equal amounts of protein extracts were loaded onto SDS-polyacrylamide gel, followed by transferring to PVDF membrane. The blot was probed with indicated primary antibody, followed by corresponding secondary antibody incubation. The blot was then developed using a chemiluminescence detection system. Blot intensity was quantified though the ImageJ software (NIH).

2.9. A549 tumor xenografts

Male nude mice (4–6 weeks old, BALB/c) were purchased from the Institute of Laboratory Animal Science, Chinese Academy of Medical Sciences (Shanghai, China). All of the experiments were performed in accordance with the Experimental Animal Research Committee of authors' affiliations. A549 cells (five million cells in 0.1 mL of culture medium) were subcutaneously injected at the right thigh of nude mice, and treatment was started when the tumors reached an average volume about 100 mm^3. Animals were randomized into two groups with 10 mice per group: (a) Vehicle; (b) 12 mg/kg of ABL. ABL was injected intraperitoneally (*i.p.*) daily. The mice were examined daily for toxicity/mortality relevant to treatment, and the tumor was measured with a caliper every two days. The tumor volume (in mm^3) was calculated by the formula: volume = $(\text{width})^2 \times \text{length}/2$, and the tumor growth curve was presented. At the end of experiments, xenograft tumors were isolated through surgery and weighted.

2.10. Immunohistochemistry (IHC) staining

The staining was performed on cryostat sections (4 μm) of xenograft tissues according to standard methods. We incubated slides in the appropriate dilution of primary antibody (1:100), and subsequently stained them with HRP-coupled secondary antibody (Santa Cruz). We visualized peroxidase activity using 3-amino-9-ethyl-carbazol (AEC) and counterstained tissues with MAYER'S solution (Merck, Shanghai, China).

2.11. Data analysis and statistics

The results were presented as mean ± standard deviation (SD). Comparisons between more than two groups were performed by analysis of variance (one-way ANOVA, SPSS 16.0). $\boldsymbol{p} < 0.05$ was considered statistically significant.

3. Results

3.1. ABL inhibits VEGF-induced HUVEC migration, proliferation and capillary structure formation

Fig. 1A demonstrated the structure of ABL. First, we tested the effect of ABL on HUVEC migration using the "Scratch" assay. Results in Fig. 1B demonstrated that ABL treatment at 5 μM or 10 μM significantly inhibited VEGF (25 ng/mL)-stimulated HUVEC migration, as the number of migrated cells decreased significantly after applied ABL treatment (Fig. 1C). Note that for the "Scratch" assay, mitomycin C was added in the culture media to prevent cell proliferation. The potential activity of ABL on HUVEC proliferation was also tested by the clonogenicity assay. Results showed that ABL treatment decreased the number of viable colonies (Fig. 1D), and its effect was dose-dependent (Fig. 1E). Meanwhile, as shown in Fig. 1F, VEGF-induced HUVEC capillary tube formation was also inhibited by ABL treatment (5 μM/10 μM), and the number of HUVEL capillary structures was significantly decreased by ABL treatment (Fig. 1G). Together, these results show that ABL inhibits HUVEC migration, proliferation and capillary structure formation.

3.2. ABL inhibits VEGF-induced angiogenesis in vivo

Next, we examined the anti-angiogenic activity of ABL using an *in vivo* model. The Matrigel plug assay was performed. After 6 days, Matrigel plugs containing VEGF appeared dark (Fig. 2A), and were filled with intact red blood cells (RBCs), indicating that functional vasculatures were formed inside the Matrigel through VEGF-induced angiogenesis (Fig. 2A). On the other hand, addition of ABL (20 μmol) in the Matrigel plugs remarkably inhibited VEGF-induced vascular formation *in vivo* (Fig. 2A). The color of the Matrigel plugs after ABL co-treatment became pale due to the lack of RBCs (Fig. 2A). Images in Fig. 2C showed that VEGF-induced microvessels formation was inhibited by ABL co-treatment *in vivo* (Fig. 2B). The number of infiltrated new, functional microvessels was decreased after ABL co-treatment (Fig. 2C). Together, these results indicate that ABL inhibits VEGF-induced angiogenesis *in vivo*.

3.3. ABL inhibits VEGF-mediated activation of Src and FAK in HUVECs

As discussed, activation of VEGFR2 and its downstream signals, *i.e.* Src kinases and FAK, plays vital roles in VEGF-mediated angiogenesis. Above results showed that ABL inhibited VEGF-induced

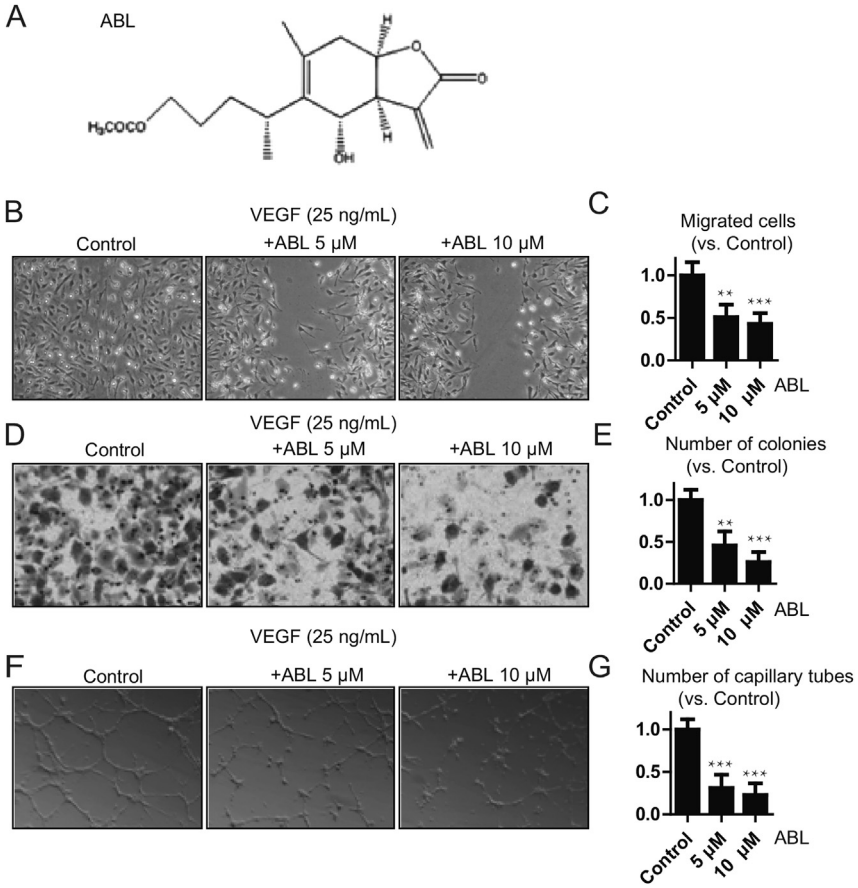

Fig. 1. The effect of ABL on HUVEC migration, proliferation and vasculogenic mimicry-The structure of ABL was presented in (**A**). HUVECs were treated with VEGF (25 ng/mL), or plus indicated concentration of ABL, cell migration was tested by "Scratch" assay (**B** and **C**, 24 h), cell proliferation was tested by clonogenicity assay (**D** and E, 10 days); the capillary tube formation was also shown (F and G, 24 h). Data were expressed as mean ± SD, experiments were repeated four times, and similar results were achieved. "Control" stands for VEGF-only group. **p < 0.01 vs. group of "Control". ***p < 0.001 vs. group of "Control".

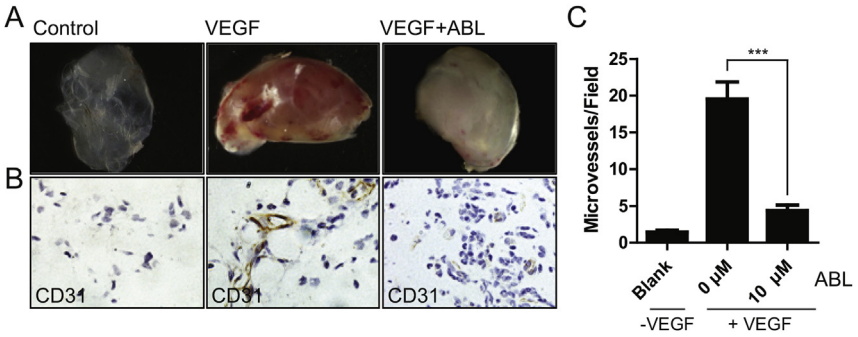

Fig. 2. The effect of ABL on VEGF-induced angiogenesis *in vivo*-C57BL/6 mice were injected with Matrigel (9 mg/mL; 0.3 mL/mouse) containing 100 ng of VEGF, 20 units of heparin, or 20 μmol of ABL into the ventral area (five mice per group). After 6 days, the skin of mice was pulled back to expose the intact Matrigel plugs, representative Matrigel plugs were photographed (**A**). The Matrigel plugs were fixed, sectioned, and stained with CD31 (**B**). Infiltrating microvessels were qualified by manual counting (**C**). Data were expressed as mean ± SD, experiments were repeated three times, and similar results were achieved. Magnification, ×200. "Control" stands for untreated blank group. ***$p < 0.001$ vs. group of "VEGF" (**C**).

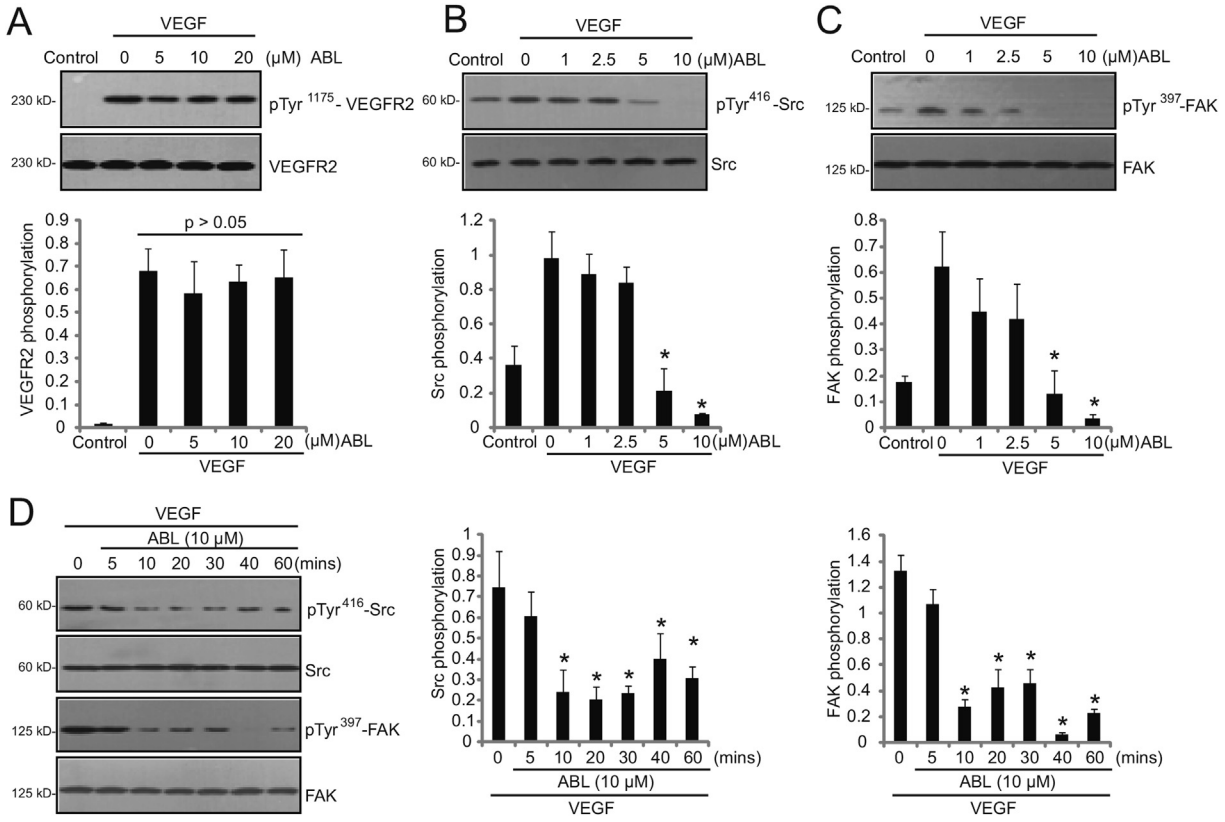

Fig. 3. The effect of ABL on VEGF signaling-HUVECs were pre-treated with applied concentration of ABL for 30 min, followed by VEGF (25 ng/mL) treatment, expressions of listed proteins were tested by Western blots (**A**–**C**). HUVECs were treated with VEGF (25 ng/mL) or plus 10 μM of ABL for indicated time, expression of listed proteins were shown (**D**). Kinase phosphorylation was quantified, results of three sets were presented. "Control" stands for untreated blank group. Data were expressed as mean ± SD. *$p < 0.05$ vs. VEGF only group.

angiogenesis both *in vitro* and *in vivo*. Its activity on VEGFR signaling was then tested. Western blot results in Fig. 3A showed that ABL at tested concentrations (5–20 μM) showed no effect on VEGFR2 phosphorylation in HUVECs. However, phosphorylation of Src and FAK, both VEGFR downstream signalings, was remarkably inhibited by ABL (Fig. 3B and C, also see quantifications). The effect of ABL was most significant at 10 μM (Fig. 3B and C, and quantification). As demonstrated in Fig. 3D, it took at least 10 min for ABL (10 μM) to exert the inhibitory activity on VEGF-induced Src-FAK signaling. These results indicate that ABL inhibits VEGF-mediated activation of Src-FAK signaling in HUVECs, which might explain its anti-angiogenesis activity.

3.4. ABL inhibits A549 cell growth in vitro

Next, we tested the potential effect of ABL on Src and FAK phosphorylation in A549 lung cancer cells. We noticed a significant high levels of Src and FAK phosphorylations in A549 cells, which were both inhibited by treatment of ABL (5 μM and 10 μM) (Fig. 4A). Src and FAK are both important for cancer cell proliferation (see discussion below). Thus, A549 cell growth, tested by MTT assay (Fig. 4B) and clonogenicity assay (Fig. 4C), was remarkably inhibited by corresponding ABL treatment. The anti-A549 cell growth activity of ABL was again dose-dependent (Fig. 4B and C). These results

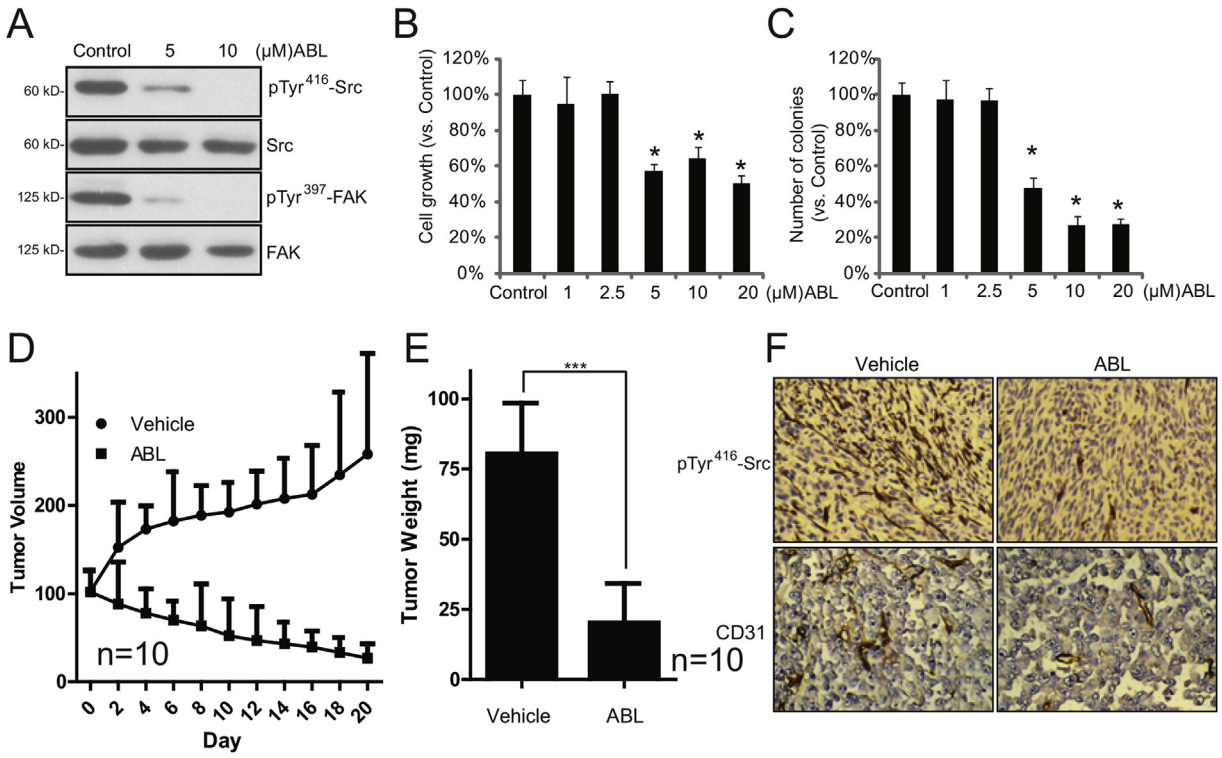

Fig. 4. ABL inhibits A549 tumor angiogenesis and growth *in vivo*-A549 cells were treated with or without indicated concentration of ABL, phosphorylated (p-) and regular Src and FAK were tested by Western blots (**A**, 4 h), cell viability was examined by MTT assay (**B**, 72 h), cell proliferation was tested by clonogenicity assay (**C**, 10 days). Male nude mice, 22–25 g, 6–8 weeks old were ear tagged and randomized into two groups (Vehicle and ABL, 12 mg/kg/day) of 10 mice each group, prior to inoculation *s.c.* with 5×10^6 A549 cells in a volume 0.1 mL culture medium. Treatment was started 14 days after inoculation. Tumor volumes (in mm^3, recorded every 2 days) were shown (**D**). At the termination of experiments (20 days after initial ABL administration), A549 xenografts were isolated and weighted (**E**), CD31 and p-Src expressions were examined by IHC staining (**F**). *In vivo* experiments were repeated twice, and similar results were obtained. Control" stands for untreated blank group (**A**–**C**). *p < 0.05 vs. group of "Control". ***p < 0.001 vs. group of Vehicle (**E**). Magnification, ×200.

demonstrate that ABL inhibits growth and Src-FAK activation in A549 lung cancer cells.

3.5. ABL inhibits A549 tumor angiogenesis and growth in vivo

To investigate the potential activity of ABL *in vivo*, a nude mice xenograft model was applied. As shown in Fig. 4D, a single dose of ABL (12 mg/kg/day, *i.p.*) dramatically inhibited the growth of A549 xenografts in nude mice. Further, the weights of ABL-treated tumors were remarkably lighter than that of vehicle-treated tumors (Fig. 4E). Notably, ABL administration didn't affect mice body weights (Data not shown). We also failed to observe any apparent toxicities or wasting in experimental animals, indicating the safety of this ABL regimen. IHC images showed that Src activation (p-Src) as well as CD31 intensity, the indicator of the tumor-supporting microvessels, were both inhibited in ABL-treated tumors (Fig. 4F). Together, these results suggest that ABL inhibits angiogenesis and A549 xenograft growth *in vivo*.

4. Discussions

In the current study, we demonstrate that ABL has significant anti-angiogenic properties *in vivo* and *in vitro*. First, we showed that ABL markedly inhibited VEGF-induced HUVEC migration, proliferation and capillary structure formation. Second, through the Matrigel plug assay, we indicated that ABL repressed VEGF-induced angiogenesis *in vivo*. Finally, we demonstrated that ABL (12 mg/kg/day) administration dramatically inhibited A549 tumor angiogenesis and growth *in vivo*. This dose failed to induce any significant toxicities or adverse effects to tested animals.

Src kinases, the largest family of non-receptor tyrosine kinases, are over-expressed and hyper-activated in lung cancers and many other malignancies [13–18]. Studies have shown that Src kinases are required for VEGF-induced angiogenesis and vascular permeability [15]. Existing evidences demonstrated that Src participates tumor angiogenesis by regulating the expression of genes of pro-angiogenic growth factors, including fibroblast growth factor (FGF), VEGF, monocyte chemotactic protein-1 (MCP-1) and interleukin-8 [13,19]. Further, Src expression promotes epithelial-to-mesenchymal transition, and is linked to solid tumor metastasis [13,19]. Meanwhile, Src expression in cancer cells is also important for cancer survival [14].

On the basis of above important functions of Src kinase, small molecule Src inhibitors have been developed, and are undergoing clinical trials [13,19]. In several of these tests, many of these Src inhibitors have displayed promising results against prostate cancer as well as other solid tumors [13,19]. Here we found that ABL dramatically inhibited VEGF-induced Src activation in HUVECs, that could explain the inhibitory effect of ABL on angiogenesis. Further, Src activation along with A549 cell proliferation were both inhibited by ABL treatment. *In vivo*, ABL administration inhibited Src phosphorylation in A549 xenografts. Thus, Src might be the primary target of ABL in inhibiting angiogenesis and cancer cell growth.

Molecularly, Src-mediated pro-cancer activities are mediated through its interactions with tyrosine kinase receptor (i.e. VEGFR), G protein-coupled receptors (GPCRs), integrins, actins, guanosine triphosphatase (GTPase)-activating proteins and non-receptor FAK [13,19,20]. FAK, just like Src kinases, also plays a major role in lung cancer angiogenesis and progression [19,20]. VEGF-mediated

activation of FAK regulates angiogenesis and various aspects of cancer progression in multiple ways [19,20]. FAK and Src connections are important for the control of cell motility and invasion [19]. Here we found that VEGF-induced FAK activation in HUVECs was dramatically inhibited ABL treatment. Further, ABL-exerted anti-A549 activity *in vitro* and *in vivo* was associated with FAK inactivation. Thus, FAK inactivation could be another important signaling mechanism of ABL-mediated inhibitory effects against angiogenesis and lung cancer cell growth. Together, these results suggest that ABL inhibits angiogenesis and lung cancer growth probably through suppressing Src-FAK signaling.

Conflicts of interests

The authors have no conflict of interests.

Acknowledgments

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