Research paper

Anlotinib inhibits angiogenesis via suppressing the activation of VEGFR2, PDGFRβ and FGFR1

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**ABSTRACT**

Angiogenesis is the sprouting and growth of new vessels from an existing vascular network. It occurs in normal development and disease such as embryogenesis and wound healing (Wang et al., 2015). Tumor angiogenesis plays an important role in tumor progress. To support their own growth and metastasis, tumors need to form new vessels to transport nutrients and oxygen (Kerbel, 2000). As such, inhibiting the formation of new vessels in tumor is a potent strategy for cancer treatment.

Previous studies indicated that tumor cells secreted pro-angiogenic cytokines to induce migration and tube formation in endothelial cells and then to generate neovascular. Vascular endothelial growth factor (VEGF), one of the most important pro-angiogenic factors, is expressed by in vast majority of cancers, especially VEGFA (Bergers and Benjamin, 2003). VEGFA has high affinity to VEGFR2 expressed in endothelial cells. The binding of VEGFA to VEGFR2 activates the tyrosine kinase receptor and the phosphorylation of VEGFR2 triggers a network of downstream pathways to promote proliferation, survival and migration of endothelial cells (Hicklin and Ellis, 2005; Claesson-Welsh and Welsh, 2013). Similar to VEGF, fibroblast growth factor 2 (FGF-2) and platelet-derived growth factor-BB (PDGF-BB) function as pro-angiogenic factors in tumor angiogenesis (Nissen et al., 2007b). PDGFR-β triggers the autophosphorylation of FGF receptor 1 (FGFR1) and activates downstream signaling cascades to induce angiogenesis (Katoh and Nakagama, 2014). PDGF-BB binds to its receptor PDGF receptor β (PDGFRβ) to regulate tumor angiogenesis, growth and metastasis (Zhao and Adjei, 2015). Now clinical strategy to target angiogenesis has achieved a qualitative progress in cancer treatment. The small-molecule tyrosine kinase inhibitors, such as sunitinib, sorafenib and nintedanib, have shown clinical efficacy in diverse cancer types (Gotink and Verheul, 2010). In this study, we found a new chemical compound (Fig. 1A), named as anlotinib, has a better anti-angiogenic effect than sunitinib, sorafenib and nintedanib. The clinical trials of anlotinib has been completed recently and is going to come into the market soon. As a TKI, anlotinib targeted multiple angiogenic kinases including VEGFR2, PDGFRβ and FGFR1. Therefore, anlotinib is a potential agent to inhibit angiogenesis and be applied to tumor therapy.

1. Introduction

Angiogenesis is the sprouting and growth of new vessels from an existing vascular network. It occurs in normal development and disease such as embryogenesis and wound healing (Wang et al., 2015). Tumor angiogenesis plays an important role in tumor progress. To support their own growth and metastasis, tumors need to form new vessels to transport nutrients and oxygen (Kerbel, 2000). As such, inhibiting the formation of new vessels in tumor is a potent strategy for cancer treatment.

Previous studies indicated that tumor cells secreted pro-angiogenic cytokines to induce migration and tube formation in endothelial cells and then to generate neovascular. Vascular endothelial growth factor (VEGF), one of the most important pro-angiogenic factors, is expressed by in vast majority of cancers, especially VEGFA (Bergers and Benjamin, 2003). VEGFA has high affinity to VEGFR2 expressed in endothelial cells. The binding of VEGFA to VEGFR2 activates the tyrosine kinase receptor and the phosphorylation of VEGFR2 triggers a network of downstream pathways to promote proliferation, survival and migration of endothelial cells (Hicklin and Ellis, 2005; Claesson-Welsh and Welsh, 2013). Similar to VEGF, fibroblast growth factor 2 (FGF-2) and platelet-derived growth factor-BB (PDGF-BB) function as pro-angiogenic factors in tumor angiogenesis (Nissen et al., 2007b). PDGFR-β triggers the autophosphorylation of FGF receptor 1 (FGFR1) and activates downstream signaling cascades to induce angiogenesis (Katoh and Nakagama, 2014). PDGF-BB binds to its receptor PDGF receptor β (PDGFRβ) to regulate tumor angiogenesis, growth and metastasis (Zhao and Adjei, 2015). Now clinical strategy to target angiogenesis has achieved a qualitative progress in cancer treatment. The small-molecule tyrosine kinase inhibitors, such as sunitinib, sorafenib and nintedanib, have shown clinical efficacy in diverse cancer types (Gotink and Verheul, 2010). In this study, we found a new chemical compound (Fig. 1A), named as anlotinib, has a better anti-angiogenic effect than sunitinib, sorafenib and nintedanib. The clinical trials of anlotinib has been completed recently and is going to come into the market soon. As a TKI, anlotinib targeted multiple angiogenic kinases including VEGFR2, PDGFRβ and FGFR1. Therefore, anlotinib is a potential agent to inhibit angiogenesis and be applied to tumor therapy.

**Abbreviations:** VEGF, vascular endothelial growth factor; FGF-2, fibroblast growth factor 2; PDGF-BB, platelet-derived growth factor-BB; VEGFR2, VEGF receptor 2; FGFR1, FGF receptor 1; PDGFRβ, PDGF receptor β; TKI, tyrosine kinase inhibitor; DMSO, dimethylsulfoxide; BSA, bovine serum albumin; CAM, Chicken Chorioallantoic Membrane Assay

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anlotinib inhibited VEGF/PDGF-BB/FGF-2-induced angiogenesis in vitro and in vivo, suggesting that anlotinib might become a potential angiogenesis inhibitor.

2. Material and method

2.1. Reagent

Anlotinib, sunitinib maleate, sorafenib tosylate, nintedanib esylate were provided by CTTQ Pharma (Lianyungang, China). The compounds were dissolved in dimethylsulfoxide (DMSO) to 10 mM as stock solution and stored at −20 °C for in vitro studies and diluted with medium before each experiment. Primary antibodies against β-actin and p-ERK were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Primary antibodies against VEGFR2, p-VEGFR2, FGFR1, p-FGFR1, PDGFRβ, p-PDGFRβ and ERK were purchased from Cell Signaling Technology (Dabvers, MA). Bovine serum albumin (BSA), Tris, NaCl, EDTA, NP-40, PMSF, NaF, SDS and DTT were purchased from Sigma (St. Louis, MO). Recombinant human vascular endothelial factor, recombinant human fibroblast growth factor 2 and recombinant human platelet-derived growth factor-BB were purchased from PeproTech (Rocky Hill, NJ). Matrigel was obtained from BD Bioscience (San Jose, CA). Transwell chamber was purchased from Merck Millipore (Billerica, MA). IRDyeTM800 conjugate secondary antibodies were come from Rockland, Inc. (Philadelphia, PA).

2.2. Cell culture

Human permanent vascular endothelial cell line, EA.hy 926 cells were purchased from Cell Bank of Shanghai Institute of Biochemistry & Cell Biology, Chinese Academy of Sciences. Cells were cultured with...
DMEM medium (Gibco, Grand Island, NY) containing 10% fetal calf serum (Gibco), 100 U/mL penicillin and 100 U/mL streptomycin and incubated in incubator with humidified atmosphere, 5% CO2 and 37 °C.

2.3. Cell viability assay

EA.hy 926 cells were seeded into 96-well plates in 10% FBS medium and the density was $1 \times 10^4$ cells. After overnight growth, treated cells with various concentration of anlotinib (0, 12.5, 25, 50, 100, 200, 400, 800, 1600 μM) in 5% CO2 incubator at 37 °C for 24 h. Added 20 μL of 0.5% MTT to the medium and incubated as previously for 4 h. Removed the supernatant and added 100 μL DMSO to 96-well plates to dissolve the precipitate. Absorbance was measured at 570 nm.

2.4. Wound healing assay

EA.hy 926 cells were seeded into six-well plates, and then wounded with a yellow pipette tip. Rinse cells with PBS (pH 7.4), add 1% FBS medium containing VEGF (20 ng/mL) (Koyama et al., 1999; Lu et al., 2008), FGF-2 (20 ng/mL) (Ettelaie et al., 2011) or PDGF-BB (100 ng/mL) (Nissen et al., 2007a). Anlotinib (0.01, 0.1 and 1 μM), sunitinib (Chinchar et al., 2014), sorafenib (Adnane et al., 2006) and nintedanib (Epstein Shochet et al., 2016) were also added into each well as indicated. The migrated distance of cells was measured and three randomly chosen fields were analyzed for each well.

2.5. Chamber directional migration assay

EA.hy 926 cells were collected at a final concentration $5 \times 10^5$ cells/mL in serum-free medium and given with various concentration of anlotinib, sunitinib, sorafenib and nintedanib. Cell suspension was added (400 μL) in the upper chamber of transwells. In the bottom chamber, 2% FBS medium containing VEGF (20 ng/mL), FGF-2 (20 ng/mL) or PDGF-BB (100 ng/mL) was added. After 6 h, fixed and stained the cells and counted the directional migration cells by microscope. Three randomly chosen fields were analyzed for each group.

2.6. Tube formation assay

EA.hy 926 cells were seeded in 6-well plates and given various concentration of anlotinib, sunitinib, sorafenib and nintedanib. After 6 h, mixed matrigel with serum-free medium (1:1), coated them on the 96-well plate (90 μL each well), solidified and polymerized at 37 °C for
30 min. EA.hy 926 cells were harvested after trypsin treatment and suspended in 1% FBS medium (control group) containing VEGF (20 ng/mL), FGF-2 (20 ng/mL) or PDGF-BB (100 ng/mL). Cell suspension was then seeded onto matrigel. After 8 h incubation, the structure of forming tubes was observed by microscope. Three randomly chosen fields were analyzed for each group.

2.7. Rat aortic ring assay

Dissect the thoracic aorta from male Sprague-Dawley rats (6 weeks old), which was cut into 1 mm long and set into 24-well plate. Clot media composed by M199+, 0.3% fibrinogen and 0.5% ε-amino-n-caproic acid (ACA, Sigma). The growth media contained M199+ with 20% FBS and 0.5% ACA. After 2 days, cells started to sprout from the explants and formed microvessel-like structures. 3 days later, choose 22 rings as experimental objects, removed the previous medium and replaced with M199+ containing 20% FBS and VEGF (50 ng/mL), FGF-2 (50 ng/mL) and PDGF-BB (50 ng/mL) respectively. Various concentrations of anlotinib, sunitinib, sorafenib and nintedanib were employed. Plates were stored in incubator at 37 °C for another 2 days and injected 10% fat emulsion (Intralipose, 10%) into embryo chorioallantois. The density and the length of vessels were observed on the surface of the CAM.

2.8. Chicken Chorioallantoic Membrane Assay (CAM)

Incubated fertilized chicken eggs were punched a small hole on the broad side and carefully created a window through the eggshell. Sterilized filter paper disks were placed on the CAM and saturated with various concentrations of anlotinib, sunitinib, sorafenib and nintedanib combined with different cytokines (200 ng/CAM). Incubated the eggs at 37 °C for another 2 days and injected 10% fat emulsion (Intralipose, 10%) into embryo chorioallantois. The density and the length of vessels were observed on the surface of the CAM.

2.9. Western blot analysis

EA.hy 926 cells were starved with serum free medium for 24 h, then treated with various concentrations of anlotinib (0.01, 0.1 and 1 μM), sunitinib, sorafenib and nintedanib. 6 h later, cells were stimulated with VEGF (20 ng/mL), FGF-2 (20 ng/mL) and PDGF-BB (100 ng/mL) respectively for 10 min. The cells were harvested and lysed in lysis buffer (50 mM Tris-Cl, pH 7.6, 150 mM NaCl, 1 mM NaF, and 1.0 mM DTT). Incubation for 1 h on ice, clarified cell lysates by centrifuging at 13,000 rpm/min for 30 min at 4 °C. Extract the supernatants and detect the concentration of protein using BCA assay with a varioskan multi-mode microplate spectrophotometer (Thermo, Waltham, MA). Separated protein containing the equal amounts by SDS-PAGE and transfer onto nitrocellulose (NC) membranes. Blocked the membrane with 1% BSA in PBS at room temperature for 1.5 h and incubated with indicated antibodies overnight at 4 °C. The next day, incubated the membrane with IRDyeTM800 conjugated secondary antibody for 1 h at room temperature. Detection was performed by the Odyssey Infrared Imaging System (LI-COR, Inc., Lincoln, NE).
2.10. Statistical analysis

All data in different experimental groups were expressed as the mean ± SEM. The data shown in the research were obtained in at least three independent experiments. Unpaired, two-tailed Student’s t-test were performed. The comparisons were made relative to cytokines-induced group and significance of difference is indicated as *P < 0.05 and **P < 0.01.

3. Result

3.1. Anlotinib inhibited VEGF/PDGF-BB/FGF-2-induced migration in EA.hy 926 cells

In order to eliminate the inhibition effect of anlotinib on the proliferation of endothelial cells, MTT assay was conducted on EA.hy 926 cells and the IC50 was 30.26 μM. Anlotinib had little effect on cell viability of EA.hy 926 cells for 24 h at the concentration of 0.01, 0.1, 1 μM. Therefore, these dosage of anlotinib were applied to the following experiments.

We detected the inhibitory of anlotinib on kinase activity and found that anlotinib worked best in inhibiting VEGFR2, PDGFRβ and FGFR1 (Fig. 1B) (Universal Kinase Activity Kit, Bio-Techne China Co. Ltd.). Therefore, their respective ligand, VEGF, PDGF-BB and FGF-2 were chose as stimulating factors in cell culture.

Wound-healing assay indicated that VEGF/PDGF-BB/FGF-2-stimulated apparent migration in EA.hy 926 cells compared with the control group after 6 h. After the treatment of anlotinib (0.01, 0.1 and 1 μM), sunitinib, sorafenib and nintedanib, less EA.hy 926 cells migrated across the plate, and the inhibitory effect of anlotinib is better than sunitinib, sorafenib and nintedanib (Figs. 2A, 4A and 6A). The inhibitory rate of anlotinib, sunitinib, sorafenib and nintedanib at 1 μM in VEGF-induced cells was 43.46%, 13.41%, 9.88% and 11.61%, respectively. Their inhibitory rate in PDGF-BB-induced cells was 66.61%, 57.96%, 32.79% and 30.67%. In FGF-2-induced cells, the inhibitory rate was 64.01%, 46.21%, 45.41% and 64.26%.

As shown in Figs. 2B, 4B and 6B, consistent with the wound healing assay, transwell assay indicated that anlotinib suppressed the direct migration of EA.hy 926 cells. The inhibitory rate of anlotinib, sunitinib, sorafenib and nintedanib at 1 μM in PDGF-BB-induced cells was 65.13%, 24.34%, 41.68% and 46.33%. In
FGF-2-induced cells, the inhibitory rate was 75.32%, 57.81%, 48.18% and 49.66%.

3.2. Anlotinib inhibited VEGF/PDGF-BB/FGF-2-induced angiogenesis in vitro and in vivo

According to the tube formation assay, VEGF/PDGF-BB/FGF-2-induced group formed much more elongated and tube-like structures than the control group. When treated with anlotinib, the formation of new tubes decreased in a concentration-dependent manner. The inhibitory effect of anlotinib at 1 μM was better than sunitinib, sorafenib and nintedanib (Figs. 3A, 5A and 7A).

As shown in Figs. 3B, 5B and 7B, the vessels sprouting of rat aortic ring were significantly induced by VEGF/PDGF-BB/FGF-2 compared with the control group. When cells were treated with different concentrations of anlotinib, the growth of new microvessels was inhibited. The inhibitory rate of anlotinib was much better than sunitinib, sorafenib, and nintedanib.

To test the effect of anlotinib on VEGF/PDGF-BB/FGF-2-induced angiogenesis in vivo, CAM assay was performed. Compared with control group (Figs. 3C, 5C and 7C), VEGF/PDGF-BB/FGF-2 induced more new blood vessels. However, anlotinib sunitinib, sorafenib and nintedanib decreased microvessel density. The inhibitory rate of anlotinib at 1 μM was higher than sunitinib, sorafenib, and nintedanib.

All these results indicated that anlotinib has a significantly inhibitory effect on angiogenesis induced by VEGF/PDGF-BB/FGF-2 in vitro and in vivo.

3.3. Anlotinib inhibited angiogenesis via blocking the activation of tyrosine kinase and their downstream signaling

We further investigated the mechanisms through which anlotinib inhibited angiogenesis. As shown in Fig. 8A, anlotinib inhibited the phosphorylation of VEGFR2 induced by VEGF and the inhibitory rate (p-VEGFR2/VEGFR2) was 40.47%, 52.19%, 56.37%. At the same time, anlotinib decreased the level of the downstream p-ERK. PDGF-BB up-regulated the level of p-PDGFRβ, however, anlotinib decreased PDGFRβ phosphorylation induced by PDGF-BB. The inhibition efficacy at 1 μM was 41.1% (Fig. 8B). Anlotinib also decreased the level of p-ERK stimulated by PDGF-BB. In addition, anlotinib significantly inhibited FGF-2-mediated FGFR1 activation and the downstream ERK phosphorylation. The inhibition rate at 1 μM was 45.0% (p-FGFR1/FGFR1). At the same time, the inhibitory effect of anlotinib on the activation of tyrosine kinases was compared with the other three drugs, sunitinib, sorafenib and nintedanib (Fig. 9). Although they all inhibited the phosphorylation of VEGFR2, PDGFRβ and FGFR1 and their downstream signaling, anlotinib showed better inhibitory effect on p-FGFR1 than the other three drugs did. According to these results, anlotinib blocked the activation of tyrosine kinase induced by their cognate cytokines and inhibited their downstream signaling.

4. Discussion

VEGF is a critical growth factor which drives tumor angiogenesis to stimulate tumor growth. High VEGF concentration correlates with poor clinical prognosis of many cancers. Inhibition of tumor angiogenesis
through pharmacological blockade of VEGF signaling is clinically approved but tumors can easily acquire drug resistance at the same time (Moens et al., 2014). VEGF stimulates the proliferation, migration, survival and new-vessel formation of endothelial cells in tumors, and blocking the action of VEGF is a proven approach to treat multiple types of solid tumors (Tredan et al., 2015). Now several VEGF-targeted agents are approved or undergoing clinical application for treatment such as bevacizumab, a VEGF-neutralizing monoclonal antibody (Ferrara et al., 2004). However, not all patients respond to bevacizumab, indicating that the treatment of bevacizumab is limited and there are other pro-angiogenic factors inducing angiogenesis besides VEGF (Mesange et al., 2014).

More and more evidence proved that there are independent roles of FGF and PDGF in tumor angiogenesis, especially without VEGF (Sun et al., 2005; Taeger et al., 2011). PDGF-BB and FGF-2 expressed and secreted by various cancer cells at high level, which could also assist VEGF to stimulate angiogenesis (Haxho et al., 2016). As a result, inhibition of angiogenic receptor tyrosine kinase has been considered as a systemic strategy for tumor treatment (Wang et al., 2017). In this research, we found that anlotinib, a multi-tyrosine kinases inhibitor, targets VEGFR2, PDGFRβ and FGFR1 kinases. Anlotinib showed a significantly inhibitory effect on angiogenesis induced by VEGF, PDGF-BB and FGF-2 in vitro and in vivo via blocking phosphorylation of three major tyrosine kinases and their downstream signaling. To test the anti-angiogenesis effect of anlotinib, we chose three FDA-approved tyrosine kinase inhibitors—sunitinib, sorafenib and nintedanib as positive drugs. In wound healing assay, chamber directional migration assay and tube formation assay, anlotinib significantly inhibits cell migration and tube formation in endothelial cells, which are the crucial steps for neovascularization. Meanwhile, anlotinib suppressed blood vessels sprout and microvessel density in rat aortic ring assay and CAM assay. Both rat aortic ring assay and CAM assay could simulate the process of angiogenesis in vivo, which further validate the overall effect of anlotinib on angiogenesis. Importantly, the inhibitory effect of anlotinib was much better than sunitinib, sorafenib and nintedanib when they were given the same concentration, suggesting that anlotinib could be a potential anti-angiogenic drug for cancer therapy.

For in vivo experiments, the LD50 of anlotinib is 1735.9 mg after 14-day oral administration, and this is far away from the treatment dosage. Anlotinib did not cause an obvious damage to liver, kidney and bone marrow. In addition, it did not show reproductive and genetic toxicity. Therefore, anlotinib shows good safety and higher bioavailability in vivo.

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**Fig. 7.** Anlotinib inhibited angiogenesis in vitro and in vivo induced by FGF-2. (A) The tube formation of EA.hy 926 cells induced by FGF-2. (B) Microvessel sprouting of rat aortic ring stimulated by FGF-2. After 3 d of microvessel-like structure growth of rat aortic ring, 20% FBS medium or FGF-2 and anlotinib were added into each group as indicated. (C) The neovessels formation in CAM. FGF-2 (200 ng/CAM) was added into exposed CAM. Sterilized filter paper disks saturated with different concentration of anlotinib was added as indicated. Samples treated with drugs and FGF-2 were compared with FGF-2 only treated group and significant of difference is indicated as *P < 0.05 and **P < 0.01.
Currently, the clinical phase III trial of anlotinib has been completed in China and anlotinib is coming into the market soon. In America, anlotinib is approved by FDA and the clinical phase II trials is underway. At present, the clinical phase II of anlotinib in thyroid medullary carcinoma, soft tissue sarcoma, renal cell carcinoma, and non-small cell lung cancer has been completed. The clinical research of anlotinib in colorectal cancer, gastric carcinoma, and esophagus cancer is ongoing and other studies in liver cancer, prostate cancer,
and neuroendocrine neoplasms are in the plans. It has been proved that anlotinib has a significant effect and favorable prognosis on advanced renal cell carcinoma, advanced non-small cell lung cancer which failed two lines of chemotherapy, medullary thyroid carcinoma and so on. We will report the effect of anlotinib on tumor patients in the future.

References


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