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Arenobufagin, a bufadienolide compound from toad venom, inhibits VEGF-mediated angiogenesis through suppression of VEGFR-2 signaling pathway

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ABSTRACT

Angiogenesis is crucial for carcinogenesis and other angiogenic processes. Arenobufagin, one of the major components of toad venom, is a traditional Chinese medicine used for cancer therapy. It inhibits cell growth in several cancer cell lines. However, little is known about arenobufagin's anti-angiogenic activity. In this study, we showed that arenobufagin inhibited vascular endothelial growth factor (VEGF)-induced viability, migration, invasion and tube formation in human umbilical vein endothelial cells (HUVECs) *in vitro*. Arenobufagin also suppressed sprouting formation from VEGF-treated aortic rings in an *ex vivo* model. Furthermore, we found that arenobufagin blocked angiogenesis in a matrigel plugs assay. Computer simulations suggested that arenobufagin interacted with the ATP-binding sites of VEGFR-2 by docking. In addition, arenobufagin inhibited VEGF-induced VEGFR-2 auto-phosphorylation and suppressed the activity of VEGFR-2-mediated signaling cascades. Taken together, our findings demonstrate that arenobufagin is a specific inhibitor of VEGF-mediated angiogenesis.

1. Introduction

Angiogenesis, the process by which new blood vessels develop from pre-existing vasculature, plays an important role in tumor growth, invasion and metastasis [1]. The angiogenic process increases the supply of nutrients, oxygen and growth factors to solid tumors, and also facilitates the removal of metabolic wastes from tumors [2]. It is believed that blocking angiogenesis could be an approach to arrest tumor growth and metastasis. Accordingly, targeting angiogenesis becomes an attractive therapeutic strategy in the treatment of cancer [3,4]. So far, a number of anti-angiogenic therapeutics have been developed in recent years, and some inhibitors have already entered into clinical application, such as bevacizumab, sunitinib and sorafenib. These angiogenesis inhibitors get good success in treating colorectal cancer, breast cancer, non-small cell lung cancer and renal cell carcinoma [5–9].

Angiogenesis is a complex process regulated by multiple growth factors and cytokines. Among these factors, VEGF is one of the most potent angiogenic factors involved in tumor growth. VEGF stimulates endothelial cell proliferation, migration and tube formation by binding to its two main receptor tyrosine kinases (RTKs) expressed on endothelial cells, VEGF receptor 1 (VEGFR-1) and VEGF receptor 2 (VEGFR-2) [10,11]. Current evidence suggests that the interaction between VEGF and VEGFR-1 plays a minor role in angiogenesis, while VEGFR-2 mediates the major angiogenic function of VEGF [11,12]. Activation of these receptors by VEGF induces the phosphorylation of a multitude of proteins in downstream signal transduction cascades, including the Erk1/2 pathway, the protein kinase C pathway, the Src family kinases and the Akt/mTOR pathway [13,14]. Therefore, VEGF and VEGFR-2 have become therapeutic targets for the development of anticancer agents. Several approaches can be taken to block VEGF/VEGFR-2 signaling pathways, such as the inhibition of endogenous VEGF release and the prevention of VEGF from binding to VEGFR-2.

Abbreviations: HUVECs, human umbilical vein endothelial cells; VEGF, vascular endothelial growth factor; VEGFR-2, vascular endothelial growth factor receptor 2; RTK, receptor tyrosine kinase; DMSO, dimethyl sulphoxide; VEGFR-1, vascular endothelial growth factor receptor 1; bFGF, basic fibroblast growth factor; Erk1/2, extracellular signal-regulated kinase; EGF, epidermal growth factor; Hsp90, heat shock protein 90; FBS, fetal bovine serum; CCK-8, cell counting kit-8; H&E, hematoxylin–eosin; Co-IP, Co- immunoprecipitation; HIF-1α, hypoxia-inducible factor-1α; FAK, Focal adhesion kinase.

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Toad venom is secreted from the postauricular and skin glands of *Bufo bufo gargarizans* Cantor or *Bufo melanostictus* Schneider, which has been used as a traditional Chinese medicine for the treatment of infection, acesodyne and cancer [15]. Arenobufagin (Fig. 1A), a bufadienolide compound, is one of the main active ingredients of toad venom. It has been reported that arenobufagin was a potent Na^+–K^+ pump inhibitor that depressed the delayed rectifier K^+ current of myocytes [16,17]. Our previous studies also found that arenobufagin suppressed human hepatoma HepG2 cell adhesion, migration and invasion [18]. However, the inhibitory activity of arenobufagin on angiogenesis has not yet been characterized.

In this study, we investigated the effects of arenobufagin on angiogenesis and explored the underlying molecular mechanisms. Our results indicated that VEGF-mediated angiogenesis was significantly inhibited by arenobufagin, suggesting that arenobufagin could be used as a potential anti-angiogenesis agent that targets VEGF/VEGFR-2 signaling pathways.

2. Materials and methods

2.1. Chemicals and reagents

Arenobufagin was isolated from the toad venom of *Bufo bufo gargarizans* by Dr. Tian Haiyan (Jinan University, Guangzhou, China). The purity of the compound was more than 99% as analyzed by high performance liquid chromatography and its chemical structure was characterized by LC–MS and NMR. The stock solution of arenobufagin was prepared in dimethyl sulphoxide (DMSO) and kept at −20 °C. Arenobufagin was diluted in culture medium to obtain the desired concentration. Arenobufagin was stable in the dilution with DMSO concentration less than 1‰.

2.2. Antibodies and other materials

Recombinant VEGF-A_{165} and basic fibroblast growth factor (bFGF) were obtained from PeproTech Company (PeproTech, Rockyhill NJ). Matrigel was obtained from BD Bioscience Company (NJ, USA). Epidermal growth factor (EGF) was obtained from Invitrogen (CA, USA). Mouse monoclonal antibodies against heat shock protein 90 β (Hsp90β), VEGF and β-actin were purchased from Santa Cruz Biotechnology (CA, USA). Rabbit polyclonal antibodies against phospho ^{Tyr1175}-VEGFR-2, phospho ^{Tyr951}-VEGFR-2, VEGFR-2, Erk1/2, phospho-Erk1/2, FAK, phospho ^{Tyr397}-FAK, Akt, phospho ^{Ser308}-Akt, phospho ^{Ser380}-PTEN, mTOR and phospho ^{Ser2448}-mTOR were purchased from Cell Signalling Technology (Danvers, MA). Rabbit polyclonal antibodies against phospho ^{Tyr529}-Src were purchased from Epitomics (Burlingame, CA). Mouse polyclonal antibodies against Hsp90α and was purchased from Abcam (Cambridge, UK). Anti-mouse and anti-rabbit IgG, (H+L) HRP-conjugate were purchased from Millipore (Billerica, MA). Normal rabbit IgG and protein A/G plus-agarose beads were obtained from Santa Cruz Biotechnology (CA, USA). Non-protein chemicals were obtained from Sigma (St Louis, MO).

2.3. Animals

Adult male Sprague-Dawley rats (weighting 220–240 g) and male C57/BL/6 mice (6 weeks old) were purchased from Guangdong Medical Laboratory Animal Center (Guangzhou, China). The animals were kept in an environmentally controlled breeding room (temperature: 25 ± 1 °C, relative humidity: 50 ± 5%, 12 h dark/light cycle from 6:00 a.m. to 6:00 p.m.), with free access to sterilized tap water and commercial laboratory rodent chow. All animal experiment procedures were conducted in accordance with institutional and Chinese government guidelines for the care and use of experimental animals.

2.4. Cell culture

Human umbilical vein endothelial cells (HUVECs) were isolated from freshly delivered umbilical cords using type II collagenase as previously described by Baudin et al. [19]. HUVECs were characterized using von Willebrand Factor VIII and CD31. HUVECs were cultured in DMEM/F12 containing 20% fetal bovine serum (FBS, Gibco), 10 μg/mL heparin, 5 ng/mL bFGF and 10 ng/mL EGF at 37 °C in a humidified atmosphere containing 5% CO_2.

2.5. Cell counting kit (CCK)-8 assay

HUVEC viability was measured using a CCK-8 kit (Dojindo, Kumamoto, Japan) [20]. Briefly, HUVECs (1×10^4 per well) were seeded in 96-well cell plates, cultured in normal growth medium (containing 20% FBS) for 24 h to obtain 80% confluent monolayer. The culture medium was replaced with normal growth medium containing arenobufagin (0, 5, 10, 50 and 125 nM). Cells were cultured and treated for 12, 24 and 48 h, respectively. Then the medium was replaced with DMEM/F12 containing 10% CCK-8. After 4 h of incubation, the absorbance was measured at 450 nm with a microplate reader (Thermo, USA). The results were calculated from five replicates of each experiment. Three independent experiments were performed.

The effect of arenobufagin on VEGF-induced cell viability was determined as previously described by Lee et al. [21]. HUVECs (1×10^4 per well) were seeded in 96-well cell plates, cultured in normal growth medium for 24 h and starved in DMEM/F12 with 5% FBS for 24 h. Cells were exposed to various concentrations of arenobufagin (0, 5, 10, 50 and 125 nM) in the presence or absence of VEGF (50 ng/mL) for 24 or 48 h in DMEM/F12 with 5% FBS. Then the medium was replaced with DMEM/F12 containing 10% CCK-8. After 4 h of incubation, the absorbance was measured at 450 nm with a microplate reader (Thermo, USA). The group without VEGF and arenobufagin treatment was set as 100%. The results were the means calculated from five replicates of each experiment. Three independent experiments were performed.

2.6. In vitro migration assay

Cell migration was assessed by the wound healing assay [22]. HUVECs were seeded in 6-well plate and allowed to grow to full confluence. Cells were firstly starved with medium containing 0.5% FBS for 6 h and wounded with pipette tips. The fresh medium (1% FBS) containing various concentrations of arenobufagin (0, 5, 10, and 50 nM), with or without 20 ng/mL VEGF was added. After 14 h of incubation, the migrated cells were photographed by using an Olympus IX70 inverted microscope (Olympus, Japan) and quantified by manual counting. The percentage of migration was the mean calculated from five replicates of each experiment. Three independent experiments were performed. The group which was not treated with VEGF and arenobufagin was set as 100%.

2.7. Invasion assay

The invasion assay was performed in Transwell (8 mm pore; Corning, Lowell, MA) pre-coated with matrigel for 8 h at 37 °C. The bottom chambers were filled with 600 μL DMEM/F12 with 1% FBS supplemented with VEGF (20 ng/mL). HUVECs (5×10^4 cells per chamber) suspended in 100 μL DMEM/F12 with 1% FBS were seeded in the top chambers. Both top and bottom chambers contained the same concentrations of arenobufagin. Cells were allowed to invade for 24 h. Non-invaded cells were scraped with