Biochemical Pharmacology 83 (2012) 1251–1260

Contents lists available at SciVerse ScienceDirect

Biochemical Pharmacology

journal homepage: www.elsevier.com/locate/biochempharm

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

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ARTICLE INFO

Article history:
Received 7 October 2011
Accepted 17 January 2012
Available online 24 January 2012

Keywords:
Arenobufagin
Angiogenesis
Vascular endothelial growth factor (VEGF)
Vascular endothelial growth factor receptor 2 (VEGFR-2)

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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doi:10.1016/j.bcp.2012.01.023

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

cotton swab on the top surface of the membrane and invaded cells were fixed with methanol and stained with Griess solution. The membrane was left to dry in the air. Images were taken using an Olympus IX70 inverted microscope, the invaded cells were counted in five independent areas per membrane. The results were the means calculated from five replicates of each experiment. Three independent experiments were performed.

2.8. Tube formation assay

Tube formation assay was carried out as described previously with some modifications [23]. A 48-well plate pre-coated with matrigel (100 μL per well) was incubated at 37 °C for 30 min. The HUVECs ($4–5 \times 10^4$) were seeded on the matrigel layer in 48-well culture plates and incubated in various concentrations of arenobufagin with or without 50 ng/mL of VEGF. After 8 h, endothelial cell tubular structures was photographed with an Olympus IX70 inverted microscope and quantified by counting the number of tubular structures in capillary networks of random five fields (control group were set as 100%) with Image-Pro Plus software (Media Cybernetics, USA). The results were the means calculated from five replicates of each experiment. Three independent experiments were carried out.

2.9. Aortic ring assay

The aortic ring assay was performed as described previously with some modifications [23]. Aortas isolated from Sprague-Dawley rats were cleaned off periadventitial fat and cut into 1–1.5 mm long rings. Aortas were rinsed, placed on the matrigel pre-coated wells, and covered with another 100 μL matrigel. Medium containing VEGF (50 ng/mL), with or without arenobufagin, was added to the wells. After 9 days, the microvessel growth was recorded with inverted microscope and the number of branching sites was quantified with Image-Pro Plus software (Media Cybernetics, USA). The results were the means calculated from five replicates of each experiment. Three independent experiments were carried out.

2.10. Matrigel plug assay

Matrigel (0.5 mL/plug) containing 250 ng VEGF and 150 units heparin with various concentrations of arenobufagin (5 or 10 μM) was injected (S.C.) into the ventral area of the 6 weeks old male C57/BL/6 mice (five mice per group). Matrigel mixed with medium alone was used as a negative control. After 21 days of implantation, the matrigel plugs were removed and the surrounding tissues were trimmed. The matrigel plugs were fixed and embedded with paraffin. Five-micron sections were stained by hematoxylin–eosin (H&E) stain. The number of blood vessels in high power field (HPF, magnification, ×200) was counted. Three independent experiments were performed.

2.11. Western blotting

HUVECs were incubated for 4 h in medium containing 4% FBS with various concentrations of arenobufagin after 6 h of serum starvation and then stimulated with VEGF (50 ng/mL) for 1 h. Whole-cell extracts were prepared using RIPA buffer supplemented with proteinase inhibitors. Proteins (50 μg) were resolved by electrophoresis on 8–12% SDS-polyacrylamide gels and were then transferred to PVDF membranes (Millipore, MA). The membrane was incubated with specific primary antibodies followed by horseradish peroxidase-conjugated goat anti-mouse or goat anti-rabbit secondary antibodies. Similar experiments were performed at least three times. The total protein concentration was determined using a BCA protein assay kit (Thermo, USA).

2.12. Molecular modeling

Docking studies were performed using the molecular modeling software package SYBYL 8.0 (Tripos, USA). The molecule was charged using the Gasteiger–Huckel and subjected to energy minimization using the Powell's method with standard Tripos force field with a 0.01 kcal/(mol Å) gradient. The minimum-energy structure was used for the docking calculations. The file 1YWN.pdb downloaded from the Protein Data Bank was used as the initial 3D structure for VEGFR-2. The crystallographic ligand was extracted from the active site, and the residues within a 6.5 Å radius around the VEGFR-2 molecule were defined as the active site. The Surflex-Dock program was used for the docking calculations with default parameters. MOLCAD surfaces were generated for visualizing the binding mode of the docked protein–ligand complexes.

2.13. Co-immunoprecipitation (Co-IP)

In order to measure the effect of arenobufagin on the interaction between VEGFR-2 and VEGF, HUVECs were incubated in DMEM/F12 with 0.5% FBS for 6 h followed by treating with arenobufagin for 4 h. Medium was replaced with DMEM/F12 containing 50 ng/mL of VEGF 1 h before harvest. Cells were then washed in ice cold PBS, and scraped into NP-40 lysis buffer containing fresh added protease inhibitors. The cell lysates were maintained constant gentle agitation for 30 min on ice and centrifuged at 4 °C, 12,000 rpm for 15 min. The supernatants were collected and the protein concentrations were measured. 1 mL cell lysate containing 1000 μg protein was precleared by adding 1 μg normal rabbit IgG and 40 μL protein A/G-Agarose beads. Together, they were incubated at 4 °C for 1 h with gentle agitation and centrifuged at 2500 rpm at 4 °C for 5 min. The supernatants were collected and subjected to immunoprecipitation with rabbit anti-VEGFR-2 antibody at a dilution of 1:200. After incubation at 4 °C for 2 h, 40 μL protein A/G-Agarose beads were added and the mixture were incubated at 4 °C for 12 h with gentle rotary agitation. The immunoprecipitates were collected by centrifugation at 2500 rpm for 5 min at 4 °C and washed with PBS for 3 times. After final wash, the immune complexes were released by boiling in 2× electrophoresis sample buffer for 5 min, followed by Western blotting analysis with rabbit anti-VEGFR-2, rabbit anti- phospho ^{Tyr1175}-VEGFR-2 and mouse anti-VEGF antibodies. Similar experiments were performed at least three times.

2.14. Statistical analysis

Data are represented as mean ± SEM. Statistical comparisons were performed using one-way analysis of variance (ANOVA) followed by a post hoc two-tailed Student's *t*-test using Origin software. p Values of <0.05 were considered statistically significant.

3. Results

3.1. Arenobufagin inhibits VEGF-induced viability of endothelial cells

The inhibitory effect of arenobufagin on cell viability in normal growth medium (containing 20% FBS) was first evaluated by CCK-8 assay. HUVECs were exposed to increasing concentrations of arenobufagin (0, 5, 10, 50 and 125 nM) for different periods of time (12, 24 and 48 h) (Fig. 1B). The results showed that arenobufagin inhibited cell viability in a dose-dependent and time-dependent manner. Significant viability inhibitory effect of arenobufagin was observed in HUVECs at concentrations more than 50 nM (Fig. 1B).

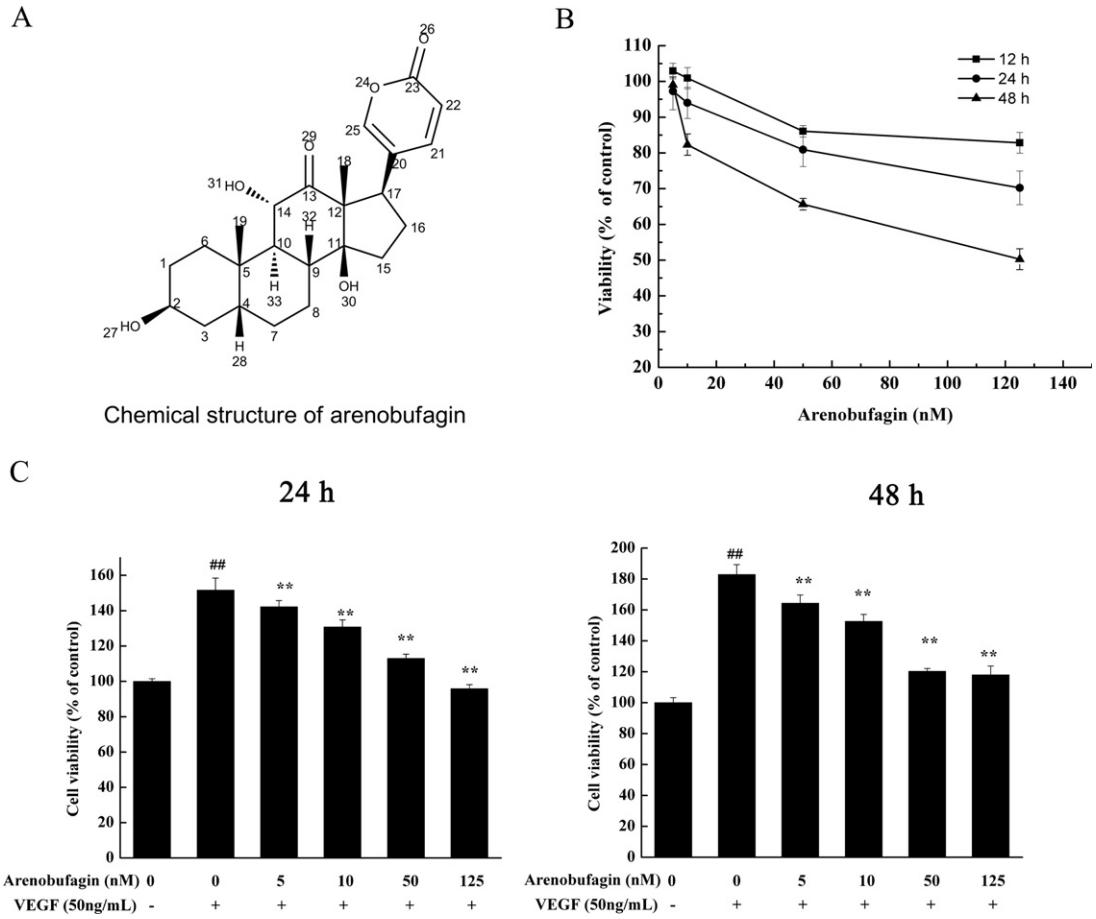

Fig. 1. Arenobufagin inhibited cell viability. (A) Chemical structure of arenobufagin. (B) Viability inhibition of arenobufagin on HUVECs under normal culture condition. HUVECs were exposed to arenobufagin in DMEM/F12 medium containing 20% FBS with heparin and growth factors for 12, 24 and 48 h and viability was measured by CCK-8 assay. Data were represented as percentage of vehicle-treated control. (C) Arenobufagin inhibited VEGF-induced cell viability in a dose-dependent manner. HUVECs were treated with arenobufagin with or without 50 ng/mL VEGF for 24 h or 48 h, and viability was measured by CCK-8 assay ($^{\#\#}p < 0.01$, VEGF-treated group vs. no VEGF-treated group; $^{**}p < 0.01$, VEGF and arenobufagin-treated group vs. VEGF-treated group).

We next studied whether arenobufagin inhibited VEGF-induced endothelial cell viability at doses ranging from 5 nM to 125 nM. Cells were seeded in 5% FBS-containing DMEM/F12 medium without growth factors, and the viability in the presence of arenobufagin for 24 or 48 h was determined after stimulation with VEGF. As shown in Fig. 1C, VEGF stimulation for 24 and 48 h increased the number of HUVECs to about 1.5 fold and 1.8 fold, respectively. Arenobufagin significantly suppressed VEGF-induced HUVECs viability in a dose-dependent manner and time-dependent manner (Fig. 1C). It was found that VEGF-activated endothelial cells were more sensitive to arenobufagin than that of in normal growth medium as indicated by the viability (Fig. 1C). Taken together, our data suggested that arenobufagin was a potent inhibitor of VEGF-induced endothelial cell viability.

3.2. Arenobufagin inhibits VEGF-induced endothelial cell migration and invasion

Because cell migration is a key step in angiogenesis, we investigated the effects of arenobufagin on the chemotactic motility of the endothelial cells by using wound-healing (Fig. 2A) assay. The results showed that arenobufagin inhibited VEGF-induced HUVECs migration in a concentration-dependent manner with significant inhibition at the dose of 50 nM.

We also performed Transwell assays to determine whether arenobufagin affected cell invasion. As shown in Fig. 2B, a large number of cells migrated to the lower side of membrane in the transwell chamber after stimulation with VEGF. However, the number of invasive cells was dramatically reduced in the presence of arenobufagin, suggestive of a potent inhibitory effect of arenobufagin on VEGF-induced endothelial cell motility.

3.3. Arenobufagin inhibits VEGF-induced endothelial cell tube formation

During the complex process of angiogenesis, the maturation of migrated endothelial cells into a capillary tube is a critical early step. Therefore, we investigated whether arenobufagin could also inhibit tube formation. As shown in Fig. 2C, HUVECs formed a robust and complete tube network in the presence of VEGF within 8 h post-seeding. However, 5 nM arenobufagin partially abolished this process with the reduction of number and length of tube-like structures, and the capillary tube was completely disassembled in the presence of 50 nM arenobufagin.

3.4. Arenobufagin inhibits VEGF-induced vessel sprouts formation

All of the above experiments revealed significant inhibitory effects of arenobufagin on a number of cellular processes in HUVECs *in vitro*. To determine whether arenobufagin also blocked angiogenesis *ex vivo*, we observed the effect of arenobufagin on the sprouting of microvessels from aortic rings. The sprouts emerged

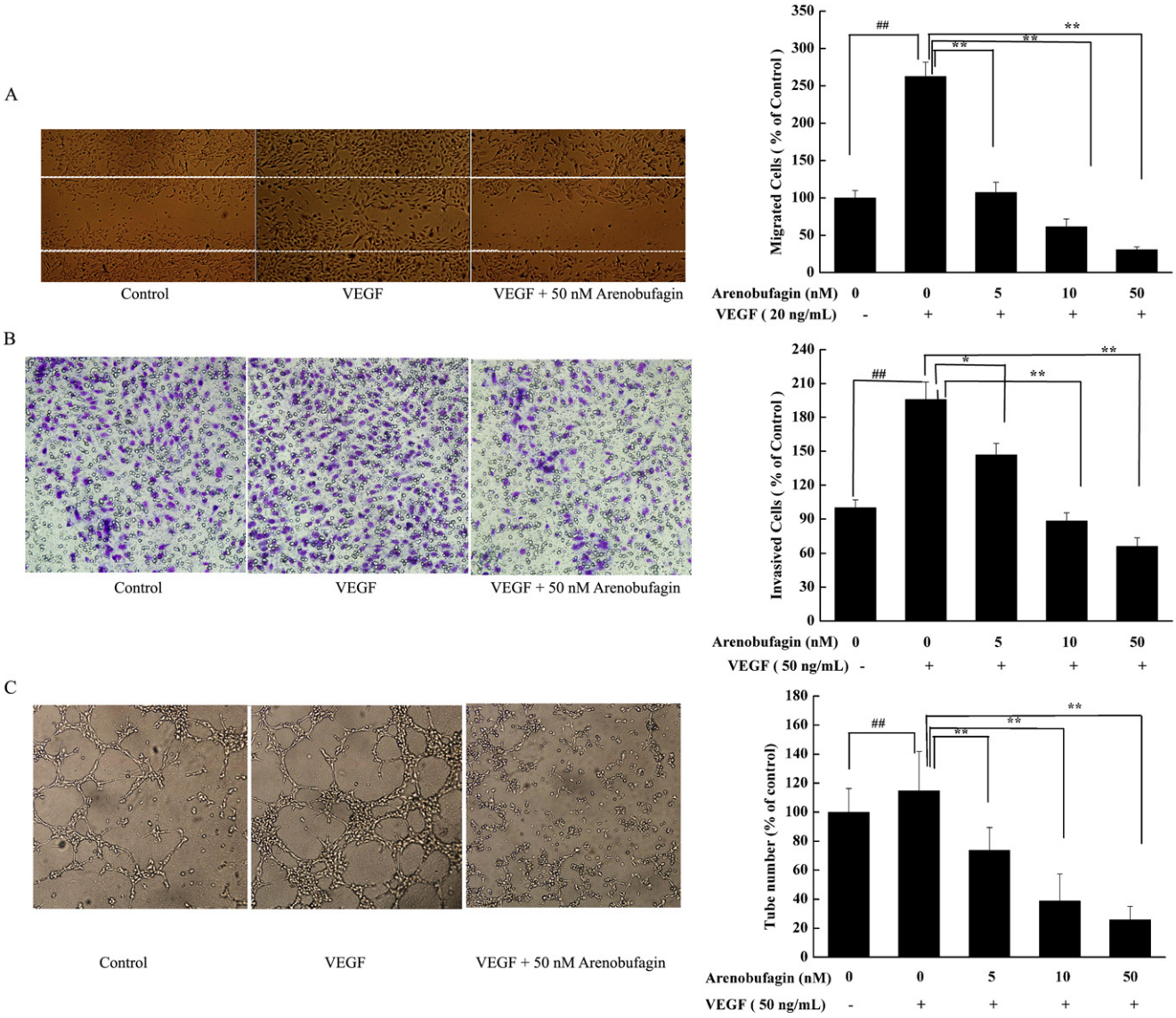

Fig. 2. Arenobufagin inhibited VEGF-induced HUVECs migration, invasion and tubulogenesis. (A) Arenobufagin inhibited VEGF-induced wound healing. HUVECs were seeded in 6-well plates and starved with medium containing 0.5% FBS for 6 h. Cell migration was measured as described in Section 2, three independent experiments were performed ($^{\#\#}p < 0.01$, VEGF-treated group vs. no VEGF-treated group; $^{**}p < 0.01$, VEGF-treated group vs. VEGF and arenobufagin-treated group). (B) Effect of arenobufagin on HUVECs invasion in Transwell assay. HUVECs were plated in Transwell pre-coated with matrigel. Cell migrated to the bottom of the membrane were counted by using an Olympus IX70 inverted microscope. Three independent experiments were performed (magnification, ×100, $^{\#\#}p < 0.01$, VEGF-treated group vs. no VEGF-treated group; $^{*}p < 0.05$; $^{**}p < 0.01$, VEGF and arenobufagin-treated group vs. VEGF-treated group). (C) Effect of arenobufagin on HUVECs tubulogenesis. HUVECs were seeded in 48-well plates pre-coated with matrigel and treated with different concentrations of arenobufagin for 8 h. Formation of cell tubular structures was photographed and quantified under an inverted microscope. Three independent experiments were performed (magnification, ×100; $^{\#\#}p < 0.01$, VEGF-treated group vs. no VEGF-treated group; $^{**}p < 0.01$, VEGF-treated group vs. VEGF and arenobufagin-treated group).

from the aortic rings and grew outward in response to 50 ng/mL VEGF while the microvessel sprouting was inhibited in a dose-dependent manner by arenobufagin (Fig. 3).

3.5. Arenobufagin inhibits VEGF-induced blood vessel formation in mice

We further investigated the activity of arenobufagin *in vivo* using the matrigel implantation assay. As shown in Fig. 4A, matrigel plugs containing VEGF excised from mice were dark red and filled with blood vessels, indicating that functional vasculatures had formed. In contrast, matrigel plugs containing both VEGF and arenobufagin were light red, suggesting that there were fewer blood vessels in the matrigel plugs. The group treated with 10 μM arenobufagin was especially pale. We then used H&E staining to quantify the number of functional vessels in the matrigel plugs (Fig. 4B). Fewer vessels were observed in the matrigel plugs treated with both VEGF and arenobufagin than in those plugs treated with VEGF alone. These results indicate that arenobufagin can inhibit angiogenesis *in vivo*.

3.6. Arenobufagin inhibits the activation of VEGFR-2 kinase in vascular endothelial cells

The above results proved that arenobufagin effectively inhibited angiogenesis both *in vitro* and *in vivo*. To understand the molecular mechanism of arenobufagin-induced inhibition of angiogenesis, we investigated whether arenobufagin suppressed phosphorylation of VEGFR-2, a critical RTK on endothelial cell surface. As shown in Fig. 5, the addition of exogenous VEGF

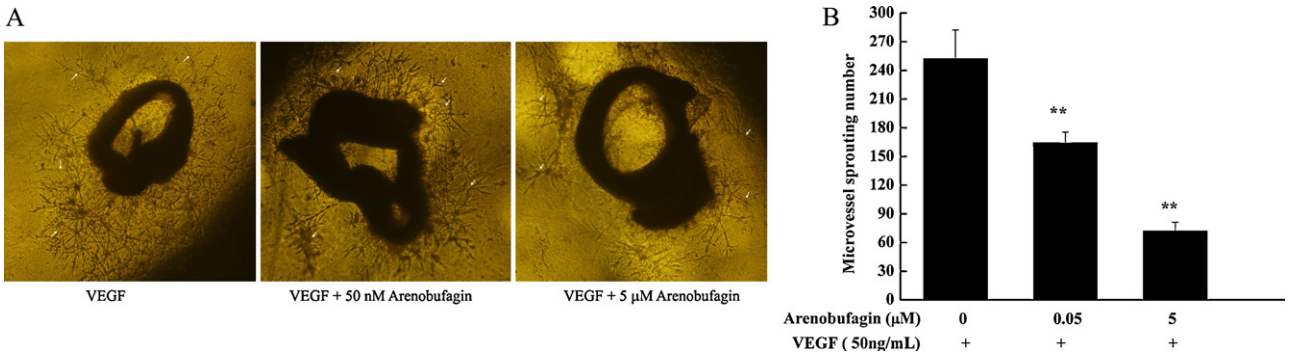

Fig. 3. Arenobufagin inhibited angiogenesis *ex vivo*. Aortic segments isolated from Sprague-Dawley rats were cultured in matrigel pre-coated wells and treated with VEGF (50 ng/ml) with or without arenobufagin for 9 days, and counted the number of capillary vessels under an Olympus IX70 inverted microscope (magnification, ×100). (A) Representative photographs of endothelial cell sprouts grown from aortic rings. (B) The number of microvessel sprouting after treatment with different concentrations of arenobufagin ($n = 5$, $^{**}p < 0.01$, VEGF-treated group vs. VEGF and arenobufagin-treated group). Three independent experiments were performed.

induced VEGFR-2 auto-phosphorylation in two different phosphorylation sites (Tyr951 and Tyr1175). Pretreatment of the cells with arenobufagin significantly blocked the activation of VEGFR-2 without affecting the overall VEGFR-2 expression level. We hypothesized that arenobufagin might bind to VEGFR-2 and subsequently affect the interaction between VEGF and VEGFR-2. To test this possibility, we carried out computer docking simulations of the interaction of arenobufagin with VEGFR-2. Molecular docking studies predicted that arenobufagin would bind at the ATP binding site of VEGFR-2. As shown in Fig. 6A, arenobufagin forms four hydrogen bonds with the ATP binding pocket of the VEGFR-2 kinase domain. The CO motif at the lactonic ring of arenobufagin forms a hydrogen bond with the backbone NH of Cys917. The OH groups at the C-2 and C-11 positions form interactions with the backbone at Phe1045 and Leu838, respectively. Additionally, the OH at the C-2 accepts a hydrogen bond with the NH residue of Lys866. The result of MOLCAD surface modeling shows that the lactonic ring of arenobufagin extends into the deep cavity of the ATP-binding pocket of VEGFR-2 (Fig. 6B).

In order to understand whether arenobufagin interfere the binding of VEGF with VEGFR-2, Co-IP experiment was then carried out to further explore the basis for VEGF-VEGFR-2 interaction. Cell lysates that were precleared by IgG were firstly immunoprecipitated with VEGFR-2 antibody and the resulting precipitates were probed with VEGF, VEGFR-2 and phospho ^{Tyr1175}-VEGFR-2 antibodies on Western blots. Both VEGF and VEGFR-2 were detected in the resulting precipitates, indicating that VEGF was associated with VEGFR-2 (Fig. 5B). The expression of phospho ^{Tyr1175}-VEGFR-2 was obviously reduced with the treatment of arenobufagin, which was consistent with the result of Western blotting analysis (Fig. 5B). The results showed that arenobufagin had little effect on VEGF-VEGFR-2 interaction, but suppressed the activation of VEGFR-2.

3.7. Arenobufagin suppresses activation of VEGFR-2-mediated signaling pathway

VEGFR-2 activation stimulates complex signaling networks with distinct and overlapping functions. A recent study suggests that the Akt/mTOR pathways and Hsp90, which are critical for angiogenesis, are phosphorylated or activated by VEGFR-2 activation in the endothelial cells [24]. In this study, we examined whether arenobufagin inhibited VEGFR-2-mediated signaling pathways. HUVECs were treated with arenobufagin for 4 h and were subsequently stimulated with 50 ng/mL of VEGF for 1 h. As shown in Fig. 7, expression levels of p-Akt and p-mTOR were greatly increased by VEGF treatment, while p-PTEN, a negative regulator of PI3K/Akt signaling pathway, was not affected by this treatment. Pretreatment of the cells with arenobufagin significantly inhibited the phosphorylation of Akt and mTOR, while the

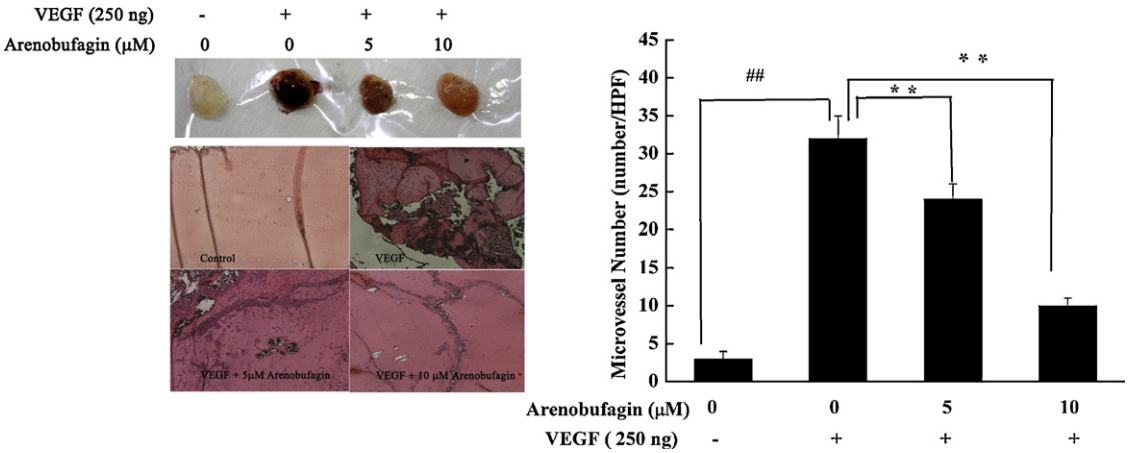

Fig. 4. Arenobufagin inhibited angiogenesis *in vivo*. (A) Photographs of the matrigel plugs. The ventral area of 6-week-old C57/BL/6 mice was injected with 500 μL of matrigel with 250 ng VEGF and 150 unit of heparin. After 21 d, the matrigel plugs were harvested. (B) Arenobufagin inhibited blood vessel formation in matrigel plugs. The matrigel plugs were fixed, sectioned and stained with H&E (magnification, ×200; $n = 5$, $^{\#\#}p < 0.01$, VEGF-treated group vs. no VEGF-treated group; $^{**}p < 0.01$, VEGF-treated group vs. VEGF and arenobufagin-treated group).

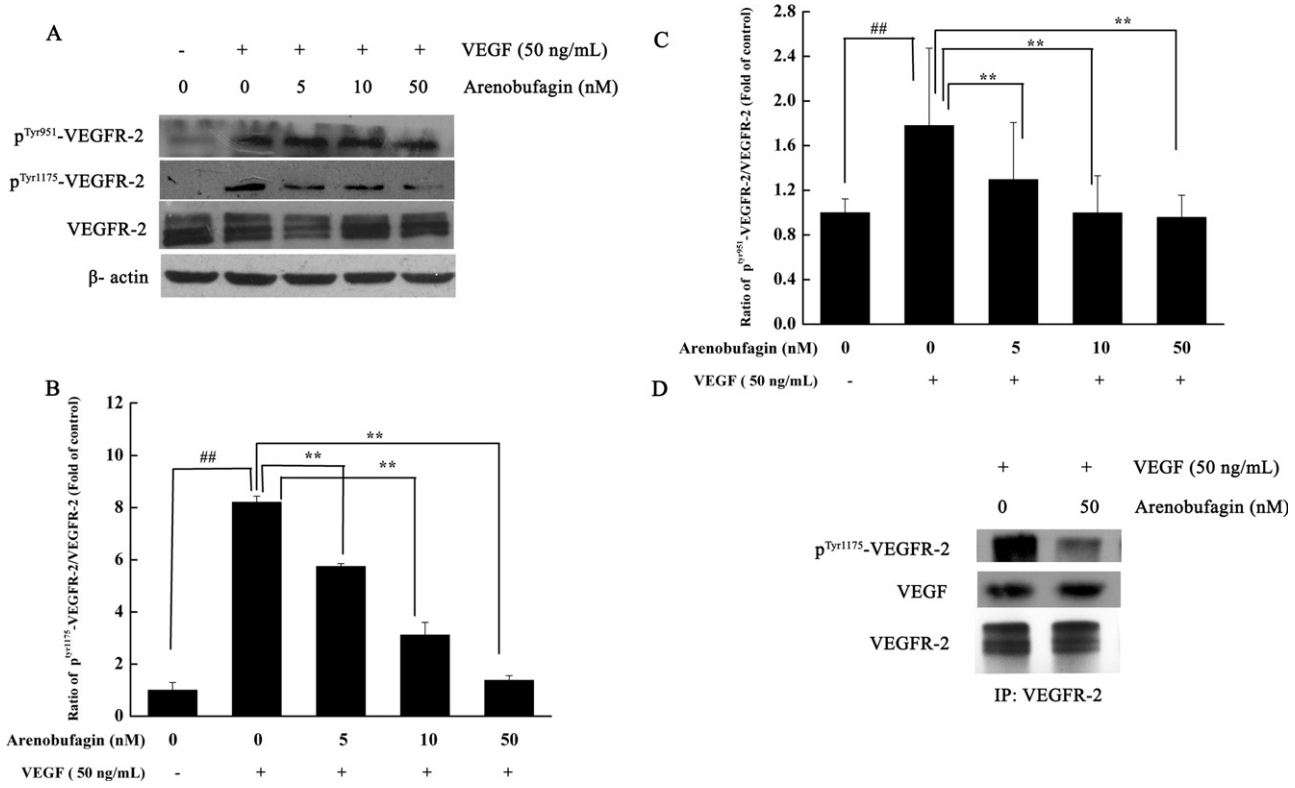

Fig. 5. Arenobufagin inhibited the phosphorylation and activation of VEGFR-2 and did not interfere with VEGF binding to VEGFR-2 in VEGF-treated HUVECs. (A) Arenobufagin inhibited the phosphorylation of VEGFR-2. The cells were starved in serum-free medium for 6 h, pretreated with various concentrations of arenobufagin for 4 h, and then stimulated with 50 ng/mL VEGF for 1 h. Protein was extracted for subsequent Western blotting analysis. (B) Ratio of phospho ^{Tyr1175}-VEGFR-2/VEGFR-2 was determined by the amount of phospho ^{Tyr1175}-VEGFR-2 normalized to the corresponding total amount of VEGFR-2 by using Gel-Pro analyzer4 (NIH, USA). The results were the means of three independent experiments ($^{\#\#}p < 0.01$ VEGF-treated group vs. no VEGF-treated group; $^{**}p < 0.01$ VEGF-treated group vs. VEGF and arenobufagin-treated group). (C) Ratio of phospho ^{Tyr951}-VEGFR-2/VEGFR-2 was determined by the amount of phospho ^{Tyr951}-VEGFR-2 normalized to the corresponding total amount of VEGFR-2 by using Gel-Pro analyzer4. The results were the means of three independent experiments ($^{\#\#}p < 0.01$ VEGF-treated group vs. no VEGF-treated group; $^{**}p < 0.01$ VEGF-treated group vs. VEGF and arenobufagin-treated group). (D) Arenobufagin did not interfere with VEGF binding to VEGFR-2. Whole-cell extracts were collected and analyzed by Co-IP assay and Western blotting using antibodies against VEGF, VEGFR-2 and p^{Tyr1175}-VEGFR-2.

total amount of Akt and mTOR remained unchanged. As expected, the level of p-PTEN did not change following arenobufagin treatment, indicating that the anti-angiogenic effects of arenobufagin are not related to p-PTEN.

Erk and Focal adhesion kinase (FAK) are two important signaling components involved in VEGF/VEGFR-2-mediated angiogenesis. The FAK and Erk pathways play key roles in endothelial cell proliferation and cell survival. Here we tested whether arenobufagin pretreatment affected the activation of the Erk pathway. The results showed that p-Erk1/2 was enhanced by VEGF treatment while the expression level of Erk1/2 remained unchanged, and that arenobufagin inhibited the phosphorylation of Erk1/2 at the concentration of 50 nM without affecting total Erk1/2 expression level (Fig. 7). Moreover, the action of arenobufagin on the phosphorylation of FAK and Src was examined. The results showed that arenobufagin inhibited the VEGF-induced phosphorylation of FAK at the dose of 10 nM and 50 nM and Src at the concentration of 50 nM (Fig. 7).

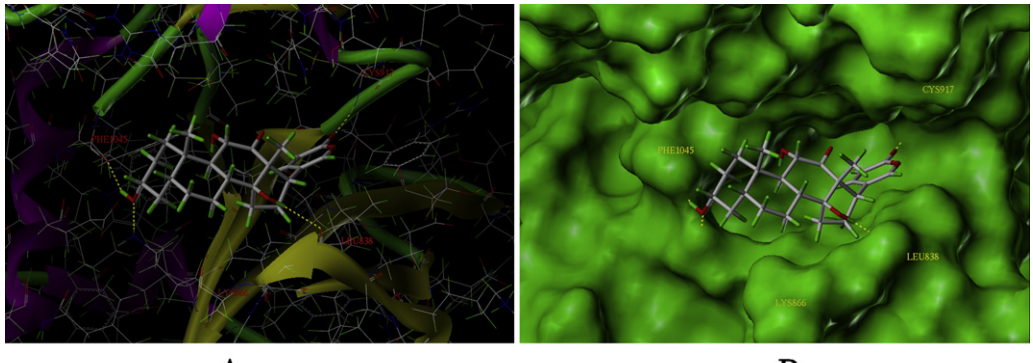

Fig. 6. The docking stereoview of arenobufagin with ATP binding site of VEGFR-2. (A) Interactions of arenobufagin and VEGFR-2 are delineated by ribbon structure. (B) MOLCAD surface representation of the ATP binding site of VEGFR-2. Hydrogen bonds are displayed as yellow dashed lines, and the participating amino acid residues are marked.

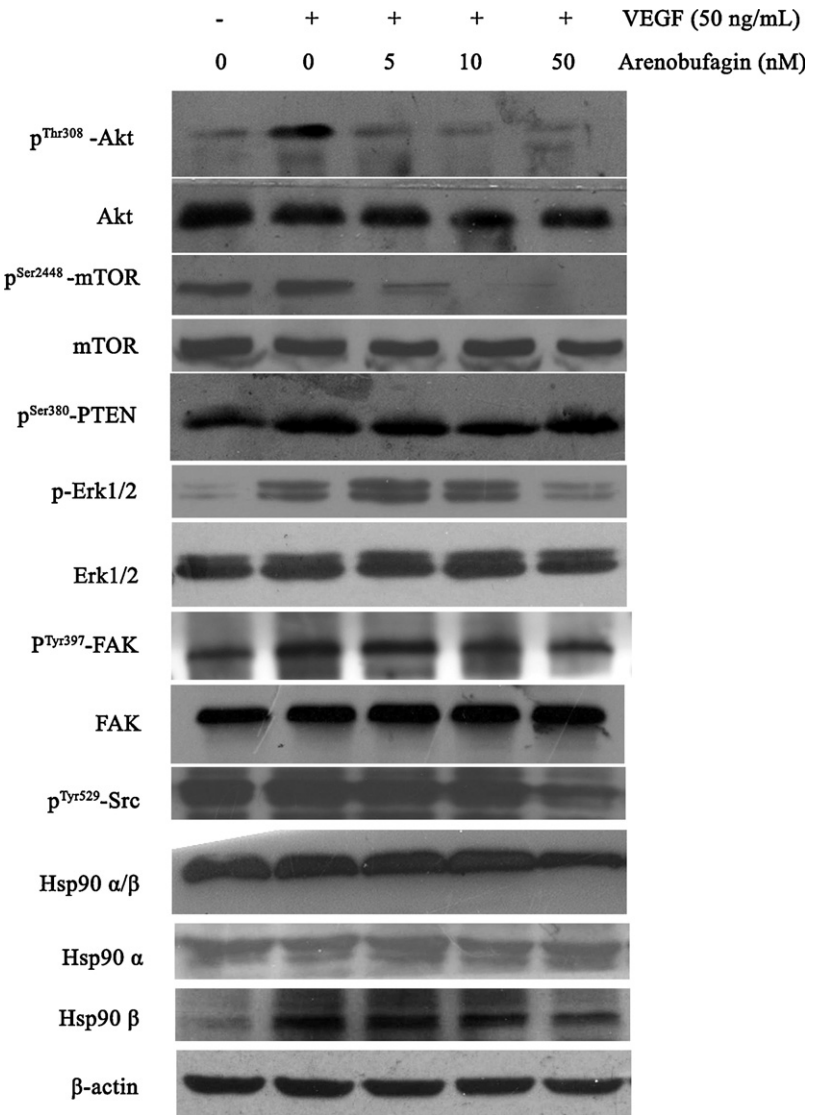

Fig. 7. Effect of arenobufagin on the expression of key proangiogenic molecules involved in VEGFR-2-mediated signaling pathway in HUVECs. HUVECs were starved in serum-free medium for 6 h, pretreated with arenobufagin for 4 h, and then stimulated with 50 ng/mL VEGF for 1 h. Protein was extracted for Western blotting analysis. Similar experiments were performed at least three times.

We further evaluated the expression level of Hsp90α/β in endothelial cells. It was found that Hsp90α/β expression was reduced by arenobufagin treatment at the concentration of 50 nM (Fig. 7). When Hsp90α and Hsp90β were analyzed separately, Hsp90β was activated by VEGF and inhibited by arenobufagin while Hsp90α was not affected, suggesting that Hsp90β but not Hsp90α contributes to the process of angiogenesis.

Taken together, our results showed that arenobufagin exerted its anti-angiogenic effect by selectively targeting certain signaling events downstream of VEGFR-2.

4. Discussion

Toad venom has been used to treat various diseases for thousands of years, but the pharmacological mechanisms of toad venom action are not fully understood. Bufalin, a digoxin-like component of toad venom, inhibits endothelial cells proliferation and tube formation [25]. Recently, bufalin and cinobufagin were reported to exhibit anti-proliferative activity and induce apoptosis of prostate cancer cells and human hepatocellular carcinoma cells via p53- and Fas-mediated apoptotic pathways [26,27]. However, research into the biological effects of the other ingredients of toad venom is limited. Arenobufagin is a key cardiotonic steroid of toad venom, but only a few studies have described its biological activities, which mostly focused on the inhibition of Na^+–K^+ ATPase [17]. One study reported that arenobufagin inhibited HepG2 cell adhesion, migration and invasion [18]. However, little is known about the inhibitory effects of arenobufagin on angiogenesis or the molecular mechanisms of these effects. Angiogenesis is important for solid tumor growth, maintenance and invasion. Tumor growth is initially fed by nearby blood vessels, and new blood vessels are required to support the growth when the tumor size exceeds a certain size [13]. Therefore, suppressing angiogenesis processes is an important strategy for cancer treatment, and the development of angiogenesis inhibitors is one important component of this goal. Here, to the best of our knowledge, we show for the first time that arenobufagin inhibits angiogenesis both *in vitro* and *in vivo*.

One of the major molecules involved in the angiogenic process is VEGF, which is secreted from tumor cells and endothelial cells. Previous studies have shown that VEGF was capable of stimulating endothelial cell proliferation, enhancing endothelial cell migration

and inducing formation of new capillaries from pre-existing vasculature, which are all key steps in the process of angiogenesis [28,29]. Thus, we studied whether arenobufagin could inhibit VEGF-induced endothelial cells viability, migration, invasion and tube formation. The activity of VEGF is mediated by its high affinity with endothelial cell receptors VEGFR-1 and VEGFR-2. In the present study, arenobufagin was shown to inhibit VEGF-induced tyrosine phosphorylation of VEGFR-2 in a dose-dependent manner. Moreover, computational docking showed that arenobufagin occupied the deep hydrophobic pocket in the ATP-binding site of VEGFR-2. In this modeling analysis, two hydroxyl groups (at the C-2 and C-11 positions of arenobufagin) bound to the ATP-binding pocket of VEGFR-2 *via* forming three hydrogen bonds. The CO motif at the lactonic ring of arenobufagin stabilized its occupation of the hydrophobic pocket of VEGFR-2. These above data suggest that arenobufagin may inhibit angiogenesis by targeting VEGFR-2. VEGFR-1 and VEGFR-2 are both RTKs and share high sequence homology. In view of this, arenobufagin might also affect VEGFR-1. However, VEGFR-1 is mostly expressed on monocytes/macrophages as well as vascular endothelium. It plays a negative role by acting as a decoy receptor or suppressing signaling through VEGFR-2 [13,30]. Even increasing number of preclinical studies validated that the therapeutic potential of soluble VEGFR-1 is an antiangiogenic agent [13]. Thus, we focus on the effect of arenobufagin on VEGFR-2 in the present study, and how arenobufagin affects VEGFR-1 is still an open question.

VEGFR-2 has strong tyrosine kinase activity, which transduces downstream signals for a series of angiogenic processes. Akt and mTOR have been proved to be involved in this growth factor receptor signaling pathway [31–34]. The Akt/mTOR pathway is critical for control of cell survival and differentiation in a variety of cell types such as endothelial cells, human breast cancer epithelial cells and smooth muscle cells [35–39]. Previous studies demonstrated that mTOR, up-regulated by hypoxia-inducible factor-1α (HIF-1α), promoted angiogenesis by enhancing VEGF transcription [33,40]. Our data showed that arenobufagin suppressed Akt phosphorylation at S308 sites in endothelial cells, suggesting Akt/mTOR pathway is involved in the inhibition of VEGF-induced endothelial cells viability.

VEGF-induced endothelial cell migration and morphogenic differentiation into capillary-like structures (*via* VEGFR-2 signaling) are two key events underlying angiogenesis. Erk1/2 and FAK play important roles in the progression of different cancers, which are important signaling effectors linking to cell adhesion, invasion, proliferation, migration and survival in many cancers [41,42]. Previous studies showed that Erk1/2 induced endothelial cell migration and was also critical for endothelial differentiation of vascular progenitor cells [43–45]. FAK in endothelial cell was induced to auto-phosphorylate at the tyrosine 397, which provides a high-affinity canonical Sh-2 binding site allowing Src to bind with [42,46]. Src–FAK interactions stimulates Src, which in turn transphosphorylate FAK on a number of key tyrosine residues and lead to the up-regulation of matrix metalloproteinases (MMPs) that contribute to the breakdown of the extracellular matrix [41,47]. We have observed here that arenobufagin inhibited HUVECs migration and formation of capillary-like structures in response to VEGF treatment, and it also inhibited the activation of Erk1/2, FAK and Src, suggesting that arenobufagin inhibits the key steps of angiogenesis, in which the Erk and FAK signaling pathways may be involved. Moreover, arenobufagin remarkably suppressed VEGF-induced new blood vessel formation in matrigel plugs implanted in mice, demonstrating that arenobufagin could inhibit angiogenesis *in vivo*.

Hsp90 is a member of the chaperone proteins family that plays an important role in the refolding of protein denatured by environmental stress [48]. Hsp90 acts on a variety of signaling proteins, including kinases and transcription factors such as Akt, Raf-1 and ErbB2 [49,50]. Importantly, Hsp90 facilitates interactions between several RTKs and their substrates, including VEGFR-2 and VEGF [51]. Previous studies showed that Hsp90 inhibition suppressed capillary tube formation in HUVECs *via* the Akt/eNOS pathway following the reduction of VEGF expression [12,52]. Therefore, inhibition of Hsp90 may be useful as a novel approach for suppressing tumor growth and angiogenesis. There are two isoforms of Hsp90 in the cytosol, Hsp90α and Hsp90β. Both of them are involved in angiogenesis and tumor metastasis [53]. An earlier study showed that Hsp90α stabilized MMP-2 in tumor cells by binding to the MMP-2 C-terminal hemopexin domain, promoting tumor angiogenesis [54]. On the other hand, Hsp90β is involved in the Bcl-2-induced activation of HIF-1α in hypoxic tumor cells, promoting HIF-1/VEGF-mediated tumor angiogenesis [55]. In present study, only Hsp90β was affected by arenobufagin treatment, suggesting that arenobufagin may inhibit angiogenesis partly through Hsp90β inactivation.

In conclusion, we provide evidence in the present study that arenobufagin suppresses HUVEC viability, migration, invasion, tube formation, and angiogenesis both *in vitro* and *in vivo*. Mechanistic investigations reveal that arenobufagin may target VEGF/VEGFR-2- mediated signaling cascades. Thus, arenobufagin could serve as a potential candidate for anti-angiogenic therapy.

Acknowledgments

We wish to thank Prof. Zhi-Jie Li, Section of Leukocyte Biology, Baylor College of Medicine, USA for the valuable comments on this manuscript. This work was supported by the National Natural Science Foundation of China (no. 90913020), Joint Fund of National Natural Science Foundation of China-Guangdong Province (no. U0932004), the Fundamental Research Funds for the Central Universities (nos. 21611314, 21611407, and 21611405).

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