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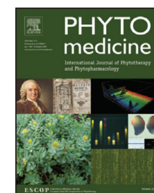

Farrerol inhibited angiogenesis through Akt/mTOR, Erk and Jak2/Stat3 signal pathway

Fujun Dai[a,1,*], Lei Gao[b,1], Yuan Zhao[a], Chaojie Wang[a], Songqiang Xie[c,*]

[a] *The Key Laboratory of Natural Medicine and Immuno-Engineering, Henan University, Kaifeng 475004, China*
[b] *Joint Tomato Research Institute, School of Agriculture and Biology, Shanghai Jiao Tong University, Shanghai 200240, China*
[c] *Institute of Chemical Biology, Pharmaceutical College of Henan University, Kaifeng 475004, China*

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ABSTRACT

Background: Farrerol is one of traditional Chinese medicines, isolated from *Rhododendron dauricum L.* It has been reported that Farrerol exerts multiple biological activities. Angiogenesis is an important drug target for cancer and inflammation therapy, the effect of Farrerol on angiogenesis is unknown.
Hypothesis/purpose: We aimed to investigate whether Farrerol may have inhibitory effects against angiogenesis.
Study design/methods: Two kinds of endothelial cells, named human umbilical vein endothelia cell and human micro vessel endothelial cells, were used to examine the effect and mechanism of Farrerol on angiogenesis. MTT assay was used to detect cell proliferation, wound healing assay and boyden's chamber assay were used to examine cell migration, Matrigel was used as basement membrane substratum in tube formation assay, Annexin V-FITC/PI dual staining assay and trypan blue staining were used to detect cell apoptosis, mouse aortic rings assay was performed as *ex vivo* assay, the expression of proteins involved in angiogenesis was tested using western blot, the binding of Farrerol to Stat3 was monitored by docking assay, molecular dynamics simulations and MM-GBSA method.
Results: Farrerol showed an inhibitory effect on proliferation, migration and tube formation of human umbilical vein endothelia cell and human micro vessel endothelial cells in a concentration-dependent manner. Farrerol induced cell cycle arrest and increased the apoptotic percentage of endothelial cells. Farrerol also suppressed the formation of new micro vessels from mouse aortic rings. Moreover, Farrerol reduced the phosphorylation levels of Erk, Akt, mTOR, Jak2 and Stat3 as well as protein expression of Bcl-2 and Bcl-xl. Docking assay, molecular dynamics simulations and MM-GBSA method showed that Farrerol bound to domain of Stat3, Ser613,Gln635, Glu638 and Thr714 are the main residues in Farrerol binding sites with the binding free energy $-7.3 \sim -9.0$ kcal/mol.
Conclusions: In this study, we demonstrated that Farrerol inhibited angiogenesis through down regulation of Akt/mTOR, Erk and Jak2/Stat3 signal pathway. The inhibitory effect of Farrerol on angiogenesis suggested that this compound may be helpful to the angiogenesis-related diseases treatment, such as cancer and inflammations.

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Introduction

Angiogenesis, considered as the process of vascular growth by sprouting from existing vessels, is a hallmark of cancers and various inflammation diseases (Carmeliet and Jain, 2000; Nagasawa et al., 2014). So, angiogenesis inhibitors are helpful to conventional therapies in treatment of these diseases. Angiogenesis is regulated by growth factors such as VEGF and FGF, several signal proteins (including Akt, Erk, mTOR) and signal transducers and activators of transcriptions (Stat)(Gupta and Qin, 2003; Munoz-Chapuli et al., 2004; Yu et al., 2014). A series of inhibitors targeting these mechanisms were applied and researched in clinical trials (Carmeliet and Jain, 2011; Kerbel and Folkman, 2002),suggesting that inhibiting the formation of new vessels is a promising therapy for aberrant angiogenesis-related diseases.

Rhododendron dauricum L. also known as 'Man shan hong', is a traditional Chinese herbal medicine with the activity of anti-chronic tracheitis and bronchitis (Xiong et al., 2013).Farrerol, a new kind of 2, 3-dihydro-flavonoid isolated from the leaves of *R.*

Abbreviations: MD, Molecular dynamics; HMEC-1, Human microvascular endothelial cell; HUVEC, Human umbilical vein endothelial cell; Stat, Signal transducers and activators of transcriptions; mTOR, Mammalian target of rapamycin.

* Corresponding authors. Tel. +86 371 22864665; fax: +86 371 22864665.
E-mail addresses: fjdwl@hotmail.com (F. Dai), xiesq@henu.edu.cn (S. Xie).
[1] Common first authors: Fujun Dai[a] and Lei Gao[b]

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dauricum L., has been to show multiple pharmacological activities such as anti-oxidant effects, anti-bacterial, anti-inflammatory effects, anti-cancer and anti-T lymphocyte activation (Ci et al., 2012; Liu et al., 2015; Qiu et al., 2011; Shi et al., 2010; Zhang et al., 2015; Zhu et al., 2007).Flavonoids are important natural compound and have been the focus of many researchers for a long time. Several flavonoids, such as nobiletin and hispidulin, had anti-angiogenic functions (Chen et al., 2015; Fotsis et al., 1997; He et al., 2011).As a new type of flavonoids, Farrerol inhibited the proliferation of rat thoracic aorta vascular smooth muscle cell (Li et al., 2011). Qingshan Li, et al demonstrated that Farrerol could protect human endothelium-derived EA.hy926 cells from apoptosis induced by hydrogen peroxide (H_2O_2) (Li et al., 2013).In addition, Farrerol also could induce apoptosis of human gastric cancer SGC-7901 cells (Liu et al., 2015). However, the effect of Farrerol on angiogenesis has not been investigated as yet.

In current study, we chose two kinds of endothelial cells, human micro vascular endothelial cells (HMEC-1) and human umbilical vein endothelial cells (HUVEC), to investigate the effect of Farrerol on angiogenesis. The results displayed that Farrerol suppressed proliferation, migration and tube formation of endothelial cells. Farrerol also induced cell cycle arrest and increased the apoptotic percentage of endothelial cells, and suppressed the formation of new microvessels sprouting from mouse aortic rings. Further studies displayed that Farrerol exerted the inhibitory effect on angiogenesis through Akt/mTOR, Erk and Jak2/Stat3 signal pathway.

Materials and methods

Regents and cell culture

Human umbilical vein endothelia cell (HUVEC) and human micro vessel endothelial cell-1 (HMEC-1) was obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA). Both cell lines were cultured in completed endothelial cell medium provided by ScienCell Research Laboratories (Carlsbad, CA,USA). The antibodies used in this study including anti-Bcl-xl, Bcl-2, Stat3, Phos-Stat3, Akt, Phos-Akt, Erk, Phos-Erk, Phos-mTOR and β-actin were purchased from Santa Cruz Biotechnology (Dallas, Texas USA). Medium was supplemented with 10% FBS, penicillin (100 units/ml) and streptomycin (100 units/ml). Farrerol ($\geq 98\%$, purity by HPLC) was obtained from Dalian meilun biology technology co., LTD (Dalian, Liaoning, China).

Wound healing migration assay

For endothelial cell migration assay, cells were seeded in 6 well plates and grown to confluency. After the starvation 4–6 h, the cells were scratched with a pipette tip followed by the addition of Farrerol. Cells were fixed using 4% paraformaldehyde after treatment for 12 h and stained with 0.2% violet crystal. Images of wound area were taken by Leica microscope (Leica, Mannheim, Germany), the number of migrated endothelial cells was counted. During the migration assays, we did not find the obvious phenomenon of apoptosis.

Transwell migration assay

Boyden's chamber assay in 24-well cell culture plate with 8-µm pore was used. Briefly, the fresh medium containing Farrerol was placed in the lower wells, and cells were suspended in the upper side of the filter at the final concentration of 4×10^4/well, and exposed to predetermined concentration of Farrerol. The plate was incubated in 37 °C and 5% CO_2 for 8 h. Cells were fixed with 4% paraformaldehyde and stained with crystal violet. Non-invaded cells on the upper side of the filter were removed by cotton swabs, and the mounted cells that invaded to the lower side of the filter were captured with an inverted microscope.

Cell proliferation assay

The function of Farrerol on cell proliferation was determined by MTT assay. In brief, HUVEC or HMEC-1 was seeded in 96-well plates (5000 cells/well). For HUVEC, the plates were coated with 0.1% Gelatin dissolved in Millipore-Q water. After culture for 12 hours, cells were starved for 6 h and subsequently treated with various concentrations of Farrerol for 48 h. Then, 50 µl of MTT solution was added to every well. After incubation for 4 h, the supernatants were discarded followed by the addition of 100 µl DMSO. The plates were vortexed for 10 min at room temperature, and the absorbances were measured with a micro plate reader (Powerwave XS; Biotek, Winooski, VT, USA).

Cell cycle and apoptosis assay

For cell cycle analysis, cells were harvested and washed with PBS twice. Then, collected cells were re-suspended in 70% cold ethanol and fixed overnight at 4 °C. After centrifugation, cells were resuspended with PBS containing RNase and stained by propidium iodide at 37 °C in dark room for 30 min. The cell cycle distribution was analyzed by flow cytometry (BD Bioscience, Franklin Lakes, NJ. USA). Annexin V-FITC/PI dual staining assay and trypan blue staining were used to detect the effect of Farrerol on apoptosis. Cells were treated with various concentrations of Farrerol for 48 h, harvested and washed with PBS twice. After centrifugation, cells were re-suspended with binding buffer and incubated with annexin V and PI. Cells were incubated in dark room at room temperature for 15 min, followed by analysis through flow cytometry. For trypan blue staining, cells were plated onto 6-well plates and were exposed to Farrerol for 48 h. After treatment, all cells including the supernatant were collected and centrifuged to collect the viable, apoptotic, and dead cells. The cells were suspended in serum-free medium. Equal volume of trypan blue and cell suspension were mixed and analyzed by an inverted microscope (Leica).

Tube formation assay

Tube formation assay was performed as described previously (Dai et al., 2012). Briefly, 96-well plates were pre-cooled followed by the addition of Matrigel. After the incubation for more than 30 min, after cell starvation for 6 h, 1×10^4 cells per well with various concentrations of Farrerol were seeded in 96-well plates. The cells were cultured at 37 °C with 5% CO_2 for 7–10 hours, tube structures in eight randomly chosen fields were photographed and counted by scoring the enclosed networks.

Mouse aortic ring assay

All animal care and experimentation conformed to the Guide for the Care and Use of Laboratory Animals published by Henan University. Thoracic aortas from Balb/c mouse were sliced into rings of 1–1.5 mm in circumference, the rings were seed into 96-well plate coated with Matrigel. Once the rings were seed onto the Matrigel, the rings were coated with Matrigel again. MCDB131 medium with or without Farrerolwas added to aortic rings coated with Matrigel. Medium with or without Farrerol was exchanged every 2 days. On day 7, micro vessel outgrowths were photographed and analyzed.

Western blot

After starvation and the subsequent treatment of Farrerol, cells were harvested and centrifuged, cell pellets were washed three

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