Rhamnazin, a novel inhibitor of VEGFR2 signaling with potent antiangiogenic activity and antitumor efficacy

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Article info
Article history:
Received 9 February 2015
Available online 19 February 2015

Keywords:
Rhamnazin
Angiogenesis
VEGFR2
Breast cancer

ABSTRACT
Anti-angiogenesis targeting vascular endothelial growth factor receptor 2 (VEGFR2) has emerged as an important tool for cancer therapy. The identification of new drugs from natural products has a long and successful history. In this study, we described a novel VEGFR2 inhibitor, rhamnazin, which inhibits tumor angiogenesis and growth. Rhamnazin significantly inhibited proliferation, migration and tube formation of human umbilical vascular endothelial cells (HUVECs) in vitro as well as inhibited sprouts formation of rat aorta ring. In addition, it inhibited vascular endothelial growth factor (VEGF)-induced phosphorylation of VEGFR2 and its downstream signaling regulator in HUVECs. Moreover, rhamnazin could directly inhibit proliferation of breast cancer cells MDA-MB-231 in vitro and in vivo. Oral administration of rhamnazin at a dose of 200 mg/kg/day could markedly inhibited human tumor xenograft growth and decreased microvessel densities (MVD) in tumor sections. Taken together, these preclinical evaluations suggest that rhamnazin inhibits angiogenesis and may be a promising anticancer drug candidate.

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1. Introduction

Worldwide, breast cancer is the most common cancer among women and has attracted global concern in recent decades [1]. It is a well-established concept that tumor-associated angiogenesis is one of the essential hallmarks underlying breast cancer development and metastasis. Thus, the anti-angiogenic therapy has become one of effective and efficient treatments for cancer [2]. During breast cancer progression, angiogenesis occurs when the total activity of pro-angiogenic molecules exceeds that of the inhibitors. After vessel invasion into breast tumor masses, there are at least six different angiogenesis-associated growth factors secreted, among which VEGF is one of the most important proangiogenic factors [3,4]. The specific action of the VEGF on the endothelial cells is mainly regulated by two types of receptor tyrosine kinases (RTKs) of the VEGF family, VEGFR1 and VEGFR2. Of the two receptors, VEGFR2 plays a more important role in mediating the mitogenesis and permeability of endothelial cells. VEGFR2 activation contributes to phosphorylation of multiple downstream signaling molecules including mitogen-activated protein kinases (MAPK), serine/threonine kinase (AKT), and signal transducer and activator of transcription 3 (STAT3) that subsequently promote tumor growth and endothelial cells proliferation, migration, and tube formation [5,6].

VEGFR2 has become an important therapeutic target for cancer anti-angiogenesis therapy. Various orally active small molecular inhibitors of VEGFR2 are now in clinical trials including sunitinib, vandetanib, and sorafenib [7]. Disappointedly, long-duration treatment with these agents might be accompanied by distinct adverse effects such as hemorrhage, hypertensive crisis, and gastrointestinal perforation [7,8]. Therefore, there has been renewed interest in natural inhibitors that could block VEGFR2 activation. Many natural products or their specific derivatives are found possessing potent anti-cancer properties [9]. In the present work, we introduced a natural compound rhamnazin [10], which is found in Nervilia fordii might, pharmacologically interfere with tumor angiogenesis. Extensive studies have reported that,
rhamnazin exerts potent anti-oxidant effects either by directly acting as an antioxidant or by activating cellular antioxidant enzyme systems [11,12]. However, the anti-angiogenesis effects of rhamnazin were not thoroughly elucidated.

In this study, the effects of rhamnazin on inhibiting angiogenesis and breast cancer cell MDA-MB-231 proliferation were validated in vitro and in vivo. Mechanistic study further indicated that rhamnazin could significantly inhibit VEGF-induced VEGFR2 phosphorylation and activation of downstream signaling transduction mediators both in vitro and in vivo. Taken together, our data suggested that rhamnazin could function as a novel potent VEGFR2 inhibitor that suppresses tumor angiogenesis and growth.

2. Materials and methods

2.1. Cell culture and rhamnazin preparation

The human breast cancer cell line (MDA-MB-231, MCF-7, T-47D, SK-BR-3, ZR-75-30) and HCT1937 was purchased from the ATCC, and maintained in L-15 medium supplemented with 10% FBS. HUVECs was purchased from Chi Scientific, and were cultivated in gelatinized culture plates in M199 medium supplemented with 15% FBS, 1% PS, 50 µg/ml endothelial cell growth supplement (ECGS, BD Bioscience) and 100 µg/ml heparin [13]. Rhamnazin (98%, Sigma ALdrich, St. Louis, MO) was dissolved in dimethyl sulfoxide (DMSO, final concentration is 0.1%) to prepare required concentrations.

2.2. One solution cell proliferation assay

The cell viability was determined by CellTiter 96® Aqueous One Solution cell proliferation assay (Promega, Madison, WI, USA). Briefly, cells were seeded in 96-well cell culture plates and treated with indicated agents. After incubation for indicated time period, 20 µL of One Solution reagent were added to each well and incubation was continued for additional 4 h. The absorbance was measured at 490 nm using Synergy™ HT Multi-Mode Microplate Reader (Bio-Tek, Winooski, VT, USA). The effect of indicated agents on cell viability was assessed as the percent of cell viability compared with vehicle-treated control cells, which were arbitrarily assigned 100% viability [14]. The concentration of rhamnazin resulting in 50% inhibition of control growth (IC50) was calculated by SPSS statistics software.

2.3. Lactate dehydrogenase (LDH) toxicity assay

The LDH released into cell cultures is an index of cytotoxicity and evaluation of the permeability of cell membrane. HUVECs were seeded in 96-well plate at a density of 3 × 10^3 cells per well. After incubation with vehicle (0.1% DMSO), 1% Triton X-100 or various concentrations of rhamnazin for 24 h, cell supernatants were collected and analyzed for LDH activity using LDH cyto-toxicity assay kit from Keygen biotech [15]. The absorbance of formed formazan was read at 490 nm on a microplate reader.

2.4. Transwell migration assay

HUVECs motility induced by 30 ng/ml recombinant Human VEGF (R&D) was evaluated in a Transwell Boyden Chamber (Corning costar) with a polycarbonate filter (8 µm pores). Briefly, Cells were added to the upper chamber containing rhamnazin, while the lower chamber contained 600 µL M199 medium containing VEGF. After incubation for 6 h, non-migrating cells were scrubbed off from the top of each filter, and cells that had migrated to the lower surface of each filter were fixed in 70% ethanol and stained with 0.1% (w/v) crystal violet solution [16]. Finally, the cells in five randomly selected microscopic fields were counted.

2.5. Tube formation assay

The tube formation assay was performed using 12-well plate coated with 100 µL Matrigel basement membrane matrix (BD Bioscience) per well and polymerized at 37 °C for 30 min. HUVECs suspended in M199 medium containing 2% FBS were plated on the Matrigel at a density of 2 × 10^5 cells/well. Rhamnazin (10, 15 and 20 µM) were then added together with VEGF. After 8 h, The Matrigel-induced morphological changes were photographed and the extent of capillary tube formation was evaluated by measuring the total tube length per field [17].

2.6. Rat aortic ring assay

Rat aortic ring assay was performed as described previously [18]. In brief, 48-well plates were coated with 120 µL of Matrigel per well and polymerized in an incubator. Aortas isolated from 6-week-old male Sprague–Dawley rats were cleaned of peri- adventitial fat and connective tissues in cold phosphate-buffered saline and cut into rings of 1–1.5 mm in circumference. The aortic rings were randomized into wells and sealed with a 100 µL overlay of Matrigel. VEGF in 500 µL of serum-free M199 with or without rhamnazin was added into the wells, and the fresh medium was exchanged for every 2 d. After 6 d, microvessel sprouting was fixed and photographed using an inverted microscope (Olympus).

2.7. Immunoprecipitation assay

HUVECs were lysed in a culture dish by adding 0.5 ml of ice-cold RIPA lysis buffer. The supernatants were collected by centrifugation at 15,000 g for 10 min at 4 °C and then incubated with IgG or VEGF in presence or absence of rhamnazin at 4 °C overnight, followed by incubation with anti-VEGFR2 for 4 h. Then, supernatants were incubation with protein G-Sepharose (Santa Cruz) for 4 h. Following the removal of supernatant by brief centrifugation (6000 g), the protein G-Sepharose were washed 3 times with lysis buffer and then boiled for 5 min in loading buffer [19]. Immunoprecipitates was further analyzed by western blotting using anti-VEGFR2 antibody and anti-VEGF antibody.

2.8. In vitro VEGFR2 kinase inhibition assay

VEGFR2 kinase assay was performed using an HTScan VEGFR2 kinase kit (CST) combined with colorimetric ELISA detection [19]. The final reaction system included 60 mM HEPES (pH 7.5), 5 mM MgCl₂, 5 mM MnCl₂, 3 mM Na3VO₄, 1.25 mM DTT, 20 µM ATP, 1.5 µM substrate peptide, 100 ng of VEGF receptor kinase, and indicated concentrations of rhamnazin.

2.9. Immunofluorescence analysis

The effects of rhamnazin on VEGF induced expression of VEGFR2 phosphorylation in HUVECs were examined using an immunocytochemical method [20]. Cells were pretreated with or without rhamnazin for 24 h in the presence of VEGF. For immunofluorescence labeling, anti-p-VEGFR2 Tyr951 antibody was used as primary antibody and goat anti-rabbit IgG-FITC was used as a secondary antibody. Fluorescence cells were observed and photographed under a
laser scanning confocal microscope (LEICA TCS SP5, Mannheim, Germany).

2.10. Western blotting assay

In brief, cell lysates were separated by 8% SDS-PAGE and transferred to polyvinylidene difluoride membranes. Membranes were then incubated with primary antibodies including phosphorylated and/or total VEGFR2, MAPK, AKT, STAT3, and GAPDH. After overnight incubation at 4 °C, membranes were incubated with secondary antibodies. Immunoreactive bands were then visualized by the enhanced chemiluminescence (ECL) detection system (GE healthcare).

2.11. Xenograft models and immunohistochemistry detections

$3 \times 10^6$ human breast cancer MDA-MB-231 cells were subcutaneously implanted into female, BALB/c nude mice to build breast cancer xenograft. Mice with appropriate size of tumors were divided randomly into two groups including vehicle-treated group and rhamnazin dosage group (200 mg/kg/day). The mice were treated with rhamnazin or carboxy methylated cellulose (vehicle) daily by intragastric administration. Tumor volume and mice body weight were measured every 3 days. Tumor volume was calculated as $\text{mm}^3 = 0.5 \times \text{length (mm)}^2 \times \text{width (mm)}$. After sacrificing mice on day 25, tumors and normal tissues will be harvested for western blotting. Band intensities were quantified using image-J software. Deparaffinized tumor sections were stained with specific antibodies including CD31 and p-VEGFR2[Y951]. Detection was done with avidin–biotin–HRP complex (Thermo scientific) and dianinobenzidine as chromogen. Nuclei were counterstained with hematoxylin. P-VEGFR2[Y951] positive cells were counted in five random high-power fields per section and were reported as a percentage of positive cells in each cellular compartment. Mean integrated optical density (mean IOD) of blood vessels accords to the following formula: mean IOD = IOD/area of the tumor section [16]. All animal experiments were carried out in compliance with the Guidelines for the Institute for Experimental Animals, Center of Nanchang University.

2.12. Statistical analysis

The data were presented as mean ± SD. Differences in the results of two groups were evaluated using either two-tailed Student’s t test or one-way ANOVA followed by post-hoc Dunnett’s test. The differences with $P < 0.05$ were considered statistically significant.

3. Results

3.1. Rhamnazin inhibited proliferation, migration and tube formation of HUVECs induced by VEGF

To assess the antiangiogenic activity of rhamnazin, we first evaluated its inhibitory effects on VEGF-induced proliferation of HUVECs. As shown in Fig. 1A, the proliferation of HUVECs stimulated by VEGF was markedly decreased after rhamnazin treatment ranging from 10 μM to 40 μM. Besides, rhamnazin had obscure inhibition effect on the proliferation of HUVECs in the absence of VEGF. To validate whether rhamnazin would result in toxicity effects on HUVECs, LDH cytotoxicity assay was carried out. As shown in Fig. 1B, Triton X-100 significantly increased LDH release and rhamnazin brought little toxic effects on HUVECs when compared to vehicle control. Endothelial cell migration is an essential step in the process of angiogenesis [21], we thus performed transwell assay to evaluate the effects of rhamnazin on HUVECs migration exposed to VEGF and observed rhamnazin strongly inhibited the migration of HUVECs (Fig. 1C). HUVECs can also spontaneously form capillary-like structures on Matrigel and so we studied the effects of rhamnazin on tube formation in HUVECs [17]. As shown in Fig. 1D, VEGF significantly increased capillary-like network. However, rhamnazin concentration-dependently decreased HUVECs tube formation in vitro. To mimic the in vivo angiogenesis situation, the organotypic assay of rat aortic ring model was built to further confirm rhamnazin inhibited VEGF-induced angiogenesis ex vivo. It was found that VEGF significantly stimulated micro vessel sprouting, leading to the formation of a network of vessels around the aortic rings. Administration rhamnazin antagonized the VEGF-induced sprouting in a dose-dependent manner, and 20 μM rhamnazin completely blocked micro-vessel sprouting of rat aortic

![Fig. 1](image-url)
rings (Fig. 1E). Together, these results indicated that rhamnazin could block VEGF-induced angiogenesis in vitro by inhibiting cell proliferation, motility and endothelial cell tubular structure formation.

3.2. Rhamnazin attenuated VEGFR-2 tyrosine kinase activity and VEGFR-2 signaling pathway

To investigate whether rhamnazin decreased the kinase activity of VEGFR2, we performed in vitro kinase assays with different concentrations of rhamnazin using HTScan® VEGFR2 kinase assay kit according to manufacturer suggested methods (Cell Signaling Technology and PerkinElmer Life Sciences, USA). Our data demonstrated that rhamnazin directly inhibited VEGFR2 kinase activity in a dose-dependent manner with an IC50 of ~4.68 μM (Fig. 2A). Immunoprecipitation-western blot analysis using HUVECs revealed that rhamnazin appeared to decrease, rather than increase VEGF binding to VEGFR2 (Fig. 2B). The phosphorylation of VEGFR2 and its downstream protein kinase stimulates angiogenesis. We investigated the effects of rhamnazin on VEGFR2 signaling pathway in HUVECs. As shown in Fig. 2C and D, rhamnazin clearly reduced VEGF-stimulated phosphorylation of VEGFR2 and its downstream MAPK, AKT, and STAT3 in HUVECs in a concentration-dependent manner [22]. In contrast, total levels of VEGFR2, MAPK, AKT, and STAT3 were not affected by rhamnazin treatment. The above results revealed that rhamnazin inhibited in vitro angiogenesis by directly targeting VEGF-VEGFR2 axis on the surface of HUVECs, and further suppressing VEGFR2 associated signaling pathways.

3.3. Rhamnazin inhibited the proliferation and VEGFR2 signaling pathway of breast cancer cells

To access the anticancer activities of rhamnazin, four human breast cancer cell lines T-47D, SK-BR-3, MCF-7 and MDA-MB-231, as well as normal (non-neoplastic) human mammary gland epithelial cells HCC1937 were used. As shown in Fig. 3A, we found rhamnazin inhibited breast cancer cell proliferation in a dose responsive manner. IC50 values from each cancer cell line and incubation time were calculated. We also noted the inhibitory effect on HCC1937...
kept at high micro-molar concentrations than the effect of equivalent doses and incubation time of rhamnazin in breast cancer cells. Collectively, these data demonstrate that rhamnazin has universal anticancer activity in breast cancer cells and especially inhibited MDA-MB-231 cells growth. As MAPK, AKT and STAT3 are reported downstream signalings after VEGFR2 activation and also involving in tumor growth [23], we detected the MAPK, AKT and STAT3 by western blot. The results showed that the VEGF-induced MAPK, AKT and STAT3 activities were significantly reduced after rhamnazin administration (Fig. 3B and C).

3.4. Rhamnazin inhibited breast cancer growth and angiogenesis in vivo

To test the anti-angiogenesis effects of rhamnazin in vivo, we utilized breast cancer xenograft model to evaluate whether rhamnazin could suppress tumor-induced angiogenesis. Prior studies demonstrated that MDA-MB-231 cell line was the often first choice as pre-clinical models for selection of targeted therapies owing to its high aggressive nature either in vitro or in vivo [24]. Thus, immunodeficient mice bearing MDA-MB-231 xenografts were treated daily with or without rhamnazin (200 mg/kg) by intragastric administration for 25 days. After treated for 25 d, the mice were sacrificed and tumor tissues were taken out for further analysis. Representative mice with MDA-MB-231 xenografts and tumor masses were shown in Fig. 4A. It was found that rhamnazin dramatically suppressed tumor volumes of the rhamnazin-treated group were inhibited by 47% compared with the vehicle group (Fig. 4B). Furthermore, rhamnazin treatment was well tolerated, and there was no significant difference in weight between vehicle group and rhamnazin treated groups (Fig. 4C).

To further examine whether rhamnazin could suppress breast cancer growth by inhibiting angiogenesis, tumor tissues were stained with specific antibodies against CD31 and p-VEGFR2Tyr951 [24]. Cluster of differentiation (CD31) is a widely used endothelial marker for quantifying angiogenesis by calculating microvessel density (MVD). Rhamnazin-treated mice showed a significant reduction of p-VEGFR2Tyr951-positive cells in tumors. Tumor sections stained with anti-CD31 antibody revealed that rhamnazin inhibited MVD (Fig. 4D). In addition, rhamnazin treatment also resulted in down-regulation of VEGFR2 downstream molecules phosphorylation including MAPK, AKT and STAT3 (Fig. 4E). All the results demonstrated that rhamnazin played an important role in suppressing angiogenesis at least partly through VEGFR2 signaling pathways.

4. Discussion

Extensive laboratory data supported that angiogenesis can be detected throughout the onset, growth, and metastasis in breast cancer. One of the initial events of angiogenesis is the secretion of multiple angiogenic factors from cancer cells. At present, VEGF has been identified as the most important pro-angiogenic factor. After binding with VEGF receptors on the surface of endothelial cell, signal pathways including MAPK/AKT will be activated, which sequentially promote endothelial cells proliferation and migration [25]. Endothelial cells proliferation plays an important role in the
process of angiogenesis from preexisting vessels. In this study, we found that rhamnazin, an O-methylated flavonol, which can be found in Rhamnus petiolaris, showed anti-proliferative effects on endothelial cells after stimulation by VEGF. Notably, we found that rhamnazin did not pose significant cytotoxicity to HUVECs at any tested concentrations based on LDH assay, indicating that the inhibitory effects of rhamnazin were not likely due to toxicity at the cellular level. Our data demonstrated that rhamnazin inhibited multiple steps of VEGF-mediated tumor angiogenesis, including cell migration and tube formation in vitro. Supporting evidences concerning anti-angiogenesis effects of rhamnazin then came from obviously inhibited sprouts formation in rat aortic ring assay.

The functions of HUVECs rely on VEGFR2 signaling and VEGFR2 phosphorylation at Tyr951 initiates downstream signaling pathways including MAPK/ATK signaling cascade. Phosphorylated MAPK and AKT are translocated into the nucleus to transmit extracellular signals that regulate cell growth, differentiation, proliferation, and migration functions [23]. Activation of the STAT3 has been shown to regulate HUVEC functions such as migration and proliferation. Inhibition of VEGFR-2 has been found to be a prosperous strategy for angiogenesis therapeutic intervention [7].

In the present study, we found that a half-maximum inhibitory concentration of 4.68 μM rhamnazin significantly blocked VEGFR2 kinase activity, making rhamnazin a potent VEGFR2 inhibitor. In addition, it was also found that rhamnazin dose-dependently inhibited VEGFR2 activity might be owing to interfering with the binding of VEGF to VEGFR2, which was validated by immunoprecipitation assay. Meanwhile, rhamnazin significantly inhibited VEGF-stimulated phosphorylation of VEGFR2 and down-stream MAPK, AKT, and STAT3 in HUVEC, indicating its ability to block angiogenesis.

Besides inhibiting tumor angiogenesis, rhamnazin also had a direct inhibitory effect on tumor cells. Rhamnazin inhibited the proliferation of MDA-MB-231 cells, which is most sensitive to rhamnazin treatment among the cancer cells treated. Rhamnazin also attenuated the VEGF-stimulated phosphorylation of MAPK, AKT, and STAT3, indicating its ability to block the oncogenic pathway. Nude mice bearing MDA-MB-231 tumor were treated daily with the vehicle or rhamnazin at 200 mg/kg/day by intraperitoneal administration. It was found that treatment with rhamnazin obviously suppressed tumor volumes, indicating that rhamnazin could significantly inhibit tumor growth in vivo. Histological studies of the tumor sections revealed that rhamnazin also significantly reduced MVD indexed by CD31 and p-VEGFR2 in comparison with vehicle group. Meanwhile, western blot results show that rhamnazin treatment could obviously attenuate expressions of p-MAPK, p-AKT, and p-STAT3 in tumor tissue, further demonstrating that rhamnazin played an important role in suppressing angiogenesis at least in part via VEGFR-2 signaling pathways in vivo.

Overall, our study indicated that rhamnazin at non-toxic dosages exerted potent anti-angiogenesis activities via specifically targeting VEGFR-2 and its signaling pathway in breast cancer. As a natural inhibitor against VEGFR-2, rhamnazin is a promising candidate for development of anti-angiogenesis agents.

Acknowledgments

National Natural Science Foundation of China (NSFC No. 81400372, 81160118, 81260113), Clinical Medicine Research Special-purpose Foundation of China (No. L2012052), Science and Technology Platform Construction Project of Jiangxi Province (No. 2013-116), Jiangxi Province Sailing Engineering (201422), Education Department Youth Scientific Research Foundation (No. GJJ14170), Youth Science Foundation of Jiangxi Province (20114BAB215036) and Science Technology Foundation of Jiangxi Province (No. 2011BBG70026-2), Health development planning commission Science Foundation of Jiangxi Province (20155154).

Conflict of interest

The authors declare that there are no conflicts of interest.
References

[22] Ding C1, C. Zhang, M. Zhang, Multitarget inhibitors derived from crosstalk mechanism involving VEGFR2, Future Med. Chem. 6 (2014) 1771–1789.