Original Article

Xanthatin, a novel potent inhibitor of VEGFR2 signaling, inhibits angiogenesis and tumor growth in breast cancer cells

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Received June 19, 2015; Accepted July 26, 2015; Epub September 1, 2015; Published September 15, 2015

Abstract: Anti-angiogenesis targeting vascular endothelial growth factor receptor 2 (VEGFR2) has emerged as an important tool for cancer treatment. In this study, we described a novel VEGFR2 inhibitor, xanthatin, which inhibits tumor angiogenesis and growth. The biochemical profiles of xanthatin were investigated using kinase assay, migration assay, tube formation, Matrigel plug assay, western blot, immunofluorescence and human tumor xenograft model. Xanthatin significantly inhibited growth, migration and tube formation of human umbilical vascular endothelial cell as well as inhibited vascular endothelial growth factor (VEGF)-stimulated angiogenesis. In addition, it inhibited VEGF-induced phosphorylation of VEGFR2 and its downstream signaling regulator. Moreover, xanthatin directly inhibit proliferation of breast cancer cells MDA-MB-231. Oral administration of xanthatin could markedly inhibit human tumor xenograft growth and decreased microvessel densities (MVD) in tumor sections. Taken together, these preclinical evaluations suggest that xanthatin inhibits angiogenesis and may be a promising anticancer drug candidate.

Keywords: Xanthatin, angiogenesis, VEGFR2, breast cancer

Introduction

Worldwide, breast cancer is one of the most common digestive malignancies and has attracted global concern in recent decades [1]. Despite considerable improvements achieved through systemic therapy, the treatment of breast cancer especially remains extremely unfavorable underscores the need for new strategies to inhibit breast cancer growth. It is a well-established concept that tumor-associated angiogenesis is one of the essential hallmarks underlying breast cancer development and metastasis. Angiogenesis is a rate-limiting process including the destabilization of integrated blood vessel, endothelial cell proliferation, migration, and tubulogenesis. 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 vascular endothelial growth factor (VEGF) is one of the most important proangiogenic factors [3]. 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 PI3K, serine/threonine kinase (Akt), and signal transducer and activator of transcription 3 (STAT3) that subsequently pro-
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mote tumor growth and endothelial cells proliferation, migration, and tube formation [4]. STAT3, a latent self-signaling transcription factor, is activated by VEGFR2. Constitutive and aberrant activation of STAT3 occurs at a frequency of 50% to 90% in a broad range of human malignancies, suggesting that STAT3 pathway is significantly associated with tumor VEGFR2 activity. 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 [5]. Disappointedly, long-duration treatment with these agents might be accompanied by distinct adverse effects such as hemorrhage, hypertensive crisis, and gastrointestinal perforation [6]. 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 [7].

Xanthatin, a natural bioactive compound of sesquiterpene lactones, was isolated and purified from air-dried aerial part of Xanthium sibiricum Patrin ex Widder. Recently, few researches demonstrate that xanthatin significantly induced cell cycle arrest and caspase-dependent apoptosis in human lung and gastric cancer, as well as murine melanoma [8, 9]. Furthermore, this natural compound shows significant cytoxicity against the HepG2 cell line and strong anti-angiogenesis capacity in vitro [10]. However, the molecular mechanism related to the anti-angiogenesis effects of xanthatin is undefined. In this study, we found that xanthatin exerted its anti-angiogenic and anti-tumor property through the suppression of VEGFR2 signaling pathway. We provide evidence that xanthatin dose dependently suppresses the activation of STAT3 in human endothelial cells. As a result, xanthatin effectively inhibited tumor angiogenesis and tumor growth in an experimental breast cancer xenograft mouse model. Therefore, our findings indicate that xanthatin is a promising candidate compound that can be further optimized to be a therapeutic agent for breast cancer.

Materials and methods

Reagents

Xanthatin (purity >98%, Sigma-Aldrich, St. Louis, MO) was dissolved in dimethyl sulfoxide (DMSO, final concentration is 0.1%) to prepare required concentrations. Recombinant human VEGF (VEGF165) was from Sino Biological Inc (11066-HNAB-20). Growth factor-reduced Matrigel was obtained from BD Biosciences. Most appropriate antibodies were purchased from Cell Signaling Technology, unless otherwise specified.

Cell culture

The human breast cancer cell line (MDA-MB-231, MCF-7, MDA-MB-415, and SK-BR-3) and HCC1937 was purchased from the ATCC, and maintained in L-15 medium supplemented with 10% FBS. HUVEC was purchased from Chi Scientific, and were cultivated in gelatinized culture plates in M199 medium supplemented with 15% FBS, 50 µg/ml endothelial cell growth supplement (ECGS, BD Bioscience) and 100 µg/ml heparin.

Cell viability assay

Briefly, HUVEC (3 × 10^4 cells per well) were seeded in 96-well plates, and exposed to various concentrations of xanthatin with VEGF (30 ng/mL) for 24 hours. Breast cancer cells (6 × 10^3 cells per well) were directly treated with xanthatin (5, 10, 15, 20, 30, and 40 µmol/L) for consecutive 24 hours. Cell viability was measured by MTT assay and three independent experiments with triplicate were carried out [11].

Lactate dehydrogenase (LDH) toxicity assay

The LDH released into cell cultures is an index of cytotoxicity and evaluation of the permeability of cell membrane. HUVEC were seeded in 96-well plate at a density of 6 × 10^3 cells per well. After incubation with vehicle (0.1% DMSO), 1% Triton X-100 or various concentrations of xanthatin for 24 h, cell supernatants were collected and analyzed for LDH activity using LDH cytotoxicity assay kit from Keygen biotech [12]. The absorbance of formed formazan was read at 490 nm on a microplate reader.

In vitro VEGFR2 kinase inhibition assay

VEGFR2 kinase assay was performed using an HTScan VEGFR2 kinase kit (CST) combined with colorimetric ELISA detection [13]. The final reaction system included 60 mM HEPES (pH 7.5), 5 mM MgCl_2, 5 mM MnCl_2, 3 µM Na_3VO_4,
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1.25 mM DTT, 20 μM ATP, 1.5 μM substrate peptide, 100 ng of VEGF receptor kinase, and indicated concentrations of xanthatin.

Migration assay

To determine the effect of xanthatin on HUVEC migration in vitro, we conducted cell migration assay by gelatin-coated Boyden inserts (8 μm; BD Biosciences). Serum-starved HUVEC (4 × 10⁴ cells) in 100 μL M199 containing 0.5% FBS were pretreated with Xanthatin (0, 5, and 10 μmol/L) for 30 minutes. Those cells were then seeded on the upper chamber of Boyden and allowed to migrate to the lower chamber with 500 μL M199 containing 0.5% FBS and 30 ng/mL VEGF. After 5 to 7 hours incubation, nonmigrated cells were removed with cotton swabs, and migrated cells were fixed with cold 4% paraformaldehyde and stained with 1% crystal violet. Images were taken with an inverted microscope (Olympus; magnification, × 100), and migrated cells in random 4 fields were quantified by manual counting. Three independent experiments with triplicate were carried out.

Endothelial cell capillary-like tube formation assay

HUVEC were pretreated with xanthatin (5 and 10 μmol/L) for 1 hour and then seeded onto the Matrigel layer in 48-well plates at a density of 4 × 10⁴ cells per well. After incubation for 6 to 8 hours, angiogenesis was assessed on the basis of formation of capillary-like structures. Tubes in randomly chosen microscopic fields were photographed (Olympus; original magnification, × 100). Three independent experiments were carried out.

In vivo Matrigel plug assay

A Matrigel plug assay was performed in BALB/c mice, as described previously with some modifications [14]. Matrigel (500 μL) containing VEGF at a final concentration of 30 ng/mL, and/or xanthatin (0, 5 and 10 μmol/L) was inoculated subcutaneously into the right flank of Balb/c mice. All treatment groups contained six mice. After 10 days, the matrigel plugs were removed and hemoglobin content was determined according to Drabkin’s method. The relative hemoglobin content was calculated versus the VEGF controls. To assay the microvascular density (MVD) of each group, HE staining was performed to visualize blood vessel.

Immunofluorescence analysis

The effects of xanthatin on VEGF induced expression of STAT3 in HUVEC were examined using an immunocytochemical method [15]. Cells were pretreated with or without xanthatin for 24 h in the presence of VEGF. For immunofluorescent labeling, anti-STAT3 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).

Western blot analysis

HUVEC were first starved in serum-free ECM for 4 hours and then pretreated with xanthatin, followed by the stimulation with 30 ng/mL VEGF. HUVEC were directly exposed to xanthatin. The whole-cell extracts were prepared by lysis buffer supplement with different kinds of protein inhibitors. Equal protein aliquot of each lysate was subjected to SDS-PAGE (12%), blotted onto polyvinylidene difluoride (PVDF) membrane (Bio-Rad), probed with specific antibodies and subsequently detected by chemiluminescence. Protein concentration was determined by Micro BCA Protein Assay Kit (Pierce Biotechnology).

Human breast tumor xenograft mouse model

Briefly, MDA-MB-231 cancer cells (4 × 10⁶ cells/mouse) were injected subcutaneously into the right flank of 6-week-old male BALB/c nude mice (National Rodent Laboratory Animal Resources, Shanghai, China). After tumor grew to about 100 mm³, the mice were randomly assigned into 2 groups (n = 8) and treated with or without xanthatin (20 mg/kg/d) via intragastric infusion for consecutive 25 days. The mice of control group were administrated with same amount of DMSO. Tumor volume and mice body weight were measured every 3 days. Tumor volume was calculated as mm³ = 0.5 × length (mm) × width (mm)² [16]. After sacrificing mice on day 25, tumors and normal tissues will be harvested for western blotting. Band intensities were quantified using image-J software. Tumor sections were stained with specific antibodies including anti-CD31 FITC, p-STAT3Ser727.
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and p-VEGFR2 Tyr951 (CST). Detection was done with avidin-biotin-HRP complex (Thermo scientific) and dianobenzidine as chromogen. Nuclei were counterstained with hematoxylin. P-VEGFR2 Tyr951 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. All animal experiments were carried out in compliance with the Guidelines for Hangzhou TCM Hospital of Zhejiang Chinese Medical University.

Statistical analysis

Statistical comparisons between groups were conducted using one-way ANOVA followed by the Dunnet test. Data were presented as means ± SDs. P values of 0.05 or less were considered statistically significant.

Results

Xanthatin inhibits VEGF-induced endothelial cell proliferation, migration, and tube formation in vitro

To systematically assess the anti-angiogenic activity of xanthatin, we first evaluated its inhibitory effects on VEGF-induced proliferation of HUVEC in vitro. As shown in Figure 1A, the proliferation of HUVEC stimulated by VEGF was markedly decreased after xanthatin treatment ranging from 10 mM to 40 mM. Besides, xanthatin had obscure inhibition effect on the proliferation of HUVEC in the absence of VEGF. To validate whether xanthatin would result in toxicity effects on HUVEC, LDH cytotoxicity assay
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was carried out. As shown in Figure 1B, Triton X-100 significantly increased LDH release and xanthatin brought little toxic effects on HUVEC when compared to vehicle control. Given the importance of vascular endothelial cell motility in the process of angiogenesis [17], we thus performed transwell assay to evaluate the effects of xanthatin on HUVEC migration exposed to VEGF and observed xanthatin remarkably inhibited the migration of HUVEC (Figure 1C). HUVEC can also spontaneously form capillary-like structures on Matrigel and so we studied the effects of xanthatin on tubulogenesis in HUVEC [18]. As shown in Figure 1D, VEGF significantly increased capillary-like network. However, xanthatin concentration-dependently decreased HUVEC tube formation in vitro.

**Xanthatin inhibits VEGF-induced angiogenesis in vivo**

To further confirm the anti-angiogenesis effects of xanthatin, matrigel plug assay was performed. As shown in Figure 2A and 2B, VEGF-induced angiogenesis in the matrigel plug was significantly inhibited by xanthatin. Relative hemoglobin content was also significantly decreased in xanthatin treated matrigel plug mice by Drabkin’s method (Figure 2C). These data indicated that, xanthatin effectively inhibited tube formation of endothelial cells and angiogenesis in the matrigel plug.

**Xanthatin blocks VEGF-induced STAT3 activation in endothelial cells**

To figure out the molecular basis of xanthatin in anti-angiogenesis, we examined the signaling pathways mediated by xanthatin in HUVEC using western blot analysis and kinase inhibition assay. VEGF signaling events relevant to tumor angiogenesis are mainly mediated by VEGF receptor 2 (VEGFR2) phosphorylation. Therefore, we first tested the action of xanthatin on this critical receptor tyrosine kinase on endothelial cell membrane. We found that xan-
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thatin significantly inhibited the phosphorylation of VEGFR2 at Tyr951 site (Figure 3A). To investigated whether xanthatin decreased the kinase activity of VEGFR2, we performed in vitro kinase assays with different concentrations of xanthatin using HTScan® VEGFR2 kinase assay kit. Our data demonstrated that xanthatin directly inhibited VEGFR2 kinase activity in a dose-dependent manner with an IC50 of ~3.8 μM (Figure 3B). Because VEGF can trigger the activation of STAT3 signaling in HUVEC, we examined the effects of xanthatin on the phosphorylation of STAT3 and showed that xanthatin dose dependently suppressed the phosphorylation of STAT3 (Ser727). When noted, such inhibitory action of xanthatin on STAT3 was in parallel with a rapid dephosphorylation of downstream kinases of STAT3, including PI3K and Akt (Figure 3C).

Additionally, the activity of STAT3 was also regulated by subcellular localization, we therefore attempted to explore the subcellular distribution of STAT3 by immunofluorescence staining and confocal microscopy. As illustrated in Figure 3D, HUVEC treatment with xanthatin (5 μM) under VEGF stimulation hardly decreased broad nuclear translocation of STAT3, while VEGF rendered STAT3 stability and general nuclear distribution. These results provide evidence that xanthatin blocked angiogenesis by targeting STAT3 signaling pathway.

**Xanthatin suppresses tumor angiogenesis and tumor growth in a human gastric cancer xenograft mouse model**

To access the anticancer activities of xanthatin, four human breast cancer cell lines MDA-MB-415, 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 Figure 4A, we found xanthatin inhibited breast cancer cell proliferation in a dose responsive manner. IC50 values from each cancer cell line and incubation time were

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**Figure 3.** Xanthatin attenuated VEGFR2 tyrosine kinase activity and VEGFR2 signaling pathway. A. Xanthatin suppressed the activation of VEGFR2 triggered by VEGF in HUVEC assayed by western blot. B. Xanthatin exhibited great inhibitory activity on VEGFR2 with an inhibitory rate of 50% at 3.8 μM. C. Xanthatin inhibited VEGFR2 downstream signaling pathways in HUVEC. Blots are representative of three experiments. Each has the expression of GAPDH as internal control. D. Immunofluorescent staining analysis of the effect of xanthatin on intracellular STAT3 expression in HUVEC. Cells were treated with xanthatin (5 μM) in the presence of VEGF. Green color was detected for STAT3, while nuclei were counterstained with blue color using DAPI (scale bar represents 50 μm).
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Figure 4. Inhibitory effects of xanthatin on tumor growth and angiogenesis. A. Breast cancer cells were exposed to indicate concentrations of xanthatin for 24 h. Cell viability was determined by MTT assay. The data are presented as mean ± SD. The values are expressed as percentage of viable cells normalized to percentage of viable cells in 0.5% DMSO-treated cells. B. Representative mice with MDA-MB-231 xenografts and tumor masses (values represent means ± SD, n = 6, **P < 0.01 versus vehicle group). C. Treatment with xanthatin resulted in significantly tumor growth inhibition versus vehicle-treated control mice. D. Body weight changes in xanthatin and vehicle treated mice. There was no significant difference in body weight between xanthatin and vehicle treated group. E. Tumor tissues were prepared for immunohistochemistry detection with antibodies against CD31, p-VEGFR2 Tyr951, and p-STAT3 Ser727 (scale bar represents 50 μm) in the tumor, with the statistical results of p-VEGFR2 Tyr951 positive cells and microvessels on the right (***P < 0.001). Data are presented as means ± SD, n = 3, *P < 0.05, **P < 0.01 versus vehicle group.
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Figure 5. Proposed model by which xanthatin treatment inhibits angiogenesis and tumor growth via inhibiting VEGFR2 signaling.

Discussion

Angiogenesis has been considered an attractive target for cancer therapy due to its pivotal role in tumor growth and metastasis. Anti-angiogenic therapies prolong the survival of cancer patients, however, without cure and at the expense of side effects [19]. VEGF has been implicated as angiogenic growth factors and identified as the most important pro-angiogenic factor. After VEGF binding with VEGF receptors on the surface of endothelial cell, signal pathways including PI3K/Akt will be activated, which sequentially promote endothelial cells growth and migration [20].

Our group has been engaged in the screening of novel angiogenesis inhibitors and in the present study, we examined a small novel molecule, xanthatin as a potent tumor angiogenesis inhibitor. In this study, we found that xanthatin showed anti-proliferative effects on endothelial cells stimulation by VEGF. Notably, we found that xanthatin did not pose significant cytotoxicity to HUVEC at any tested concentrations based on LDH assay, indicating that the inhibitory effects of xanthatin was not likely due to toxicity at the cellular level. Our data demonstrated that xanthatin inhibited multiple steps of VEGF-mediated tumor angiogenesis, including cell migration and tube formation in vitro. Supporting evidences concerning anti-angiogenesis effects of xanthatin then came from obviously inhibited sprouts formation in Matrigel plug assay.

The functions of HUVEC rely on VEGFR2 signaling and VEGFR2 phosphorylation at Tyr951 initiates downstream PI3K/Akt signaling cascade. Phosphorylated PI3K and Akt are translocated into the nucleus to transmit extracellular signals that regulate endothelial cells proliferation, migration and angiogenesis [20]. Activation of the STAT3 has been shown to regulate HUVEC functions and regulates PI3K/Akt activity. In the present study, we found that a half-maximum inhibitory concentration of 3.8 μM xanthatin significantly blocked VEGFR2 kinase activity, making xanthatin a potent VEGFR2 inhibitor. Meanwhile, xanthatin significantly inhibited VEGF-stimulated phosphorylation of VEGFR2 and down-stream STAT3, PI3K, and Akt in HUVEC, indicating its ability to block angiogenesis.
Besides inhibiting angiogenesis, xanthatin also inhibited the proliferation of MDA-MB-231 cells, which is most sensitive to xanthatin treatment among all the cancer cell lines treated. Nude mice bearing MDA-MB-231 tumor were treated with the vehicle or xanthatin (20 mg/kg/day). It was found that treatment with xanthatin obviously suppressed tumor volumes, indicating that xanthatin could significantly inhibit tumor growth in vivo. Histological studies of the tumor sections revealed that xanthatin also significantly reduced MVD indexed by anti-CD31 FITC, p-VEGFR2[tyr951], p-STAT3[ser727] and in comparison with vehicle group. Meanwhile, xanthatin treatment could obviously attenuate expressions of p-STAT3 in tumor tissue, further demonstrating that xanthatin played an important role in suppressing angiogenesis at least in part via STAT3 signaling pathways in vivo (Figure 5). Overall, our study indicated that xanthatin at non-toxic dosages exerted potent anti-angiogenesis activities via specifically targeting VEGFR2 and its signaling pathway in breast cancer. As a natural inhibitor against VEGFR2 and angiogenesis, xanthatin is a promising candidate for development of anti-angiogenesis agents.

Acknowledgements

National Natural Science Foundation of China (81160118 and 81400372); Clinical Medicine Research Special-purpose Foundation of China (L2012052); Jiangxi Province Voyage Project (2014022); Jiangxi province Degree and Postgraduate Education Reform Project (2015); Science and Technology Platform Construction Project of Jiangxi Province (2013116); Youth Science Foundation of Jiangxi Province (2015-1BAB215016); Technology and Science Foundation of Jiangxi Province (20151BBG0223); Jiangxi Province Education Department Scientific Research Foundation (GJJ14170); Health Development Planning Commission Science Foundation of Jiangxi Province (20155154); Scholar Project of Ganjiang River (2015).

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