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Anti-angiogenic Effect of Ginsenoside Rh2 by Downregulation of VEGF in a Zebrafish Model

MA Liman[1], QI Yongxiao[1], Wang Wenji[2]* and Zhong Qianyi[3]*

[1]*School of Medicine, Taizhou University, Taizhou, Zhejiang 318000, P.R. China.*
[2]*School of Life Science, Taizhou University, Taizhou, Zhejiang 318000, P.R. China.*
[3]*Taizhou Central Hospital, Taizhou, Zhejiang 318000, P.R. China.*

ABSTRACT

Angiogenesis, the formation of new blood vessels, is tightly regulated in the normal organism. Excessive angiogenesis can have detrimental consequences in various processes, including promoting uncontrolled tumor growth. Inhibiting angiogenesis using natural compounds is an important focus in developing new cancer treatment strategies. Ginsenoside Rh2 (G-Rh2), a natural product derived from red ginseng, has been reported to have therapeutic effects on some tumors, but its antiangiogenic effects in zebrafish has not been evaluated. Here we investigated the antiangiogenic of G-Rh2 using the zebrafish angiogenesis model. Morphological observations of the G-Rh2-treated wild-type embryos were performed to evaluate the toxicity of the compound. Tg(*fli1-EGFP*) transgenic zebrafish with fluorescent blood vessels were studied to evaluate vascular development defects. G-Rh2 treatment up to 84.85 μM did not induce morphological defects in zebrafish. We observed suppression effect of angiogenesis by G-Rh2 in inhibiting intersegmental vessel formation from 30 to 84.85 μM in a dose-dependent manner. Moreover, quantitative real-time PCR revealed that G-Rh2 suppressed vascular endothelial growth factor (*vegf*) mRNA expression but not mRNA expression of its receptors (*flt1*, *kdr*). Western blot further confirmed that G-Rh2 treatment resulted in reduced Vegf protein expression. Our study suggested that G-Rh2 may exert anti-angiogenic activity by downregulation of Vegf in zebrafish embryos, thus indicating its role as a potential therapeutic agent against cancer.

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Authors' Contribution
ML and WW designed the experiments. QY and ZQ raised fish and collected the samples. ML executed the experimental work and worte the article.

Key words
Angiogenesis, Ginsenoside Rh2, Vegf, Zebrafish

INTRODUCTION

Angiogenesis is a physiological process in which new blood vessels emerge from preexisting vessels (Wong *et al*., 2009; Berthod, 2014). Mounting evidence has demonstrated that angiogenesis plays an important role in tumor progression (Kondo *et al*., 2002; Mantovani, 2010). Therefore, inhibition of the tumor angiogenesis system has become a vital strategy for cancer therapy (Jansen *et al*., 2004; Dong *et al*., 2007). The vascular endothelial growth factor (VEGF) is one of the most important activators of angiogenesis and acts by binding to its two tyrosine kinase receptors *VEGFR-1* (*FLT1*) and *VEGFR-2* (*FLK1*, *KDR*), which are expressed primarily in endothelial cells (Ferrara *et al*., 2003). Several reports suggested that inhibition of the VEGF pathway is the best-validated therapeutic approach in the cancer treatment (Jain, 2005; Jain *et al*., 2006; Crawford and Ferrara, 2009). A recombinant humanized anti-VEGF monoclonal antibody (bevacizumab) has been approved by the Food and Drug Administration for first-line treatment of several cancers (Yang *et al*., 2003; Sandler *et al*., 2006; Saltz *et al*., 2008). Tyrosine kinase inhibitors targeting the VEGF receptor are also being evaluated in clinical studies (Matulonis *et al*., 2009; Natale *et al*., 2009).

Identifying antiangiogenic drugs from natural compounds holds great promise for cancer therapy. Ginseng is a widely used Chinese herb that is associated with a significantly decreased risk of a variety of cancers (Jin *et al*., 2016). Ginsenoside Rh2 (G-Rh2) is one of the key components isolated from red ginseng with potential bioactivity. G-Rh2 potently induces cellular apoptosis in hepatoma and leukemia Reh cells (Park *et al*., 2012; Xia *et al*., 2014). In addition, G-Rh2 exerts anti-cancer activities on tumor growth and metastasis in prostate cancer (Zhang *et al*., 2015; Huang *et al*., 2019), colorectal cancer (Han *et al*., 2016), and other malignant tumors (Nakata *et al*., 1998; Shi *et al*., 2014). G-Rh2 inhibited tumor angiogenesis and growth in a Lewis pulmonary carcinoma mouse model (Cui and Qu, 2011). However, whether G-Rh2 exhibits antiangiogenic activity in zebrafish embryos and its underlying mechanism have not been fully investigated.

The zebrafish, *Danio rerio*, has emerged as an excellent model for studying a wide variety of biological processes (Thisse and Zon, 2002; He *et al*., 2014). The advantages of studying angiogenesis in zebrafish include

* Corresponding author: wangwj@tzc.edu.cn; zhongqy@tzzxyy.com
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its external fertilization, early optical clarity and easy genetic manipulation (Santoro, 2014). Moreover, the effects of several anti-angiogenic drugs examined in the zebrafish model were also observed in mammalian systems (Cross *et al*., 2003). Additionally, the availability of transgenic zebrafish that express enhanced green fluorescent protein (GFP) in the vasculature enables the visualization of vascular defects in live zebrafish embryos (Tran *et al*., 2007). Previous studies have examined G-Rh2 in various cells and models; however, the effects of G-Rh2 in the zebrafish model have not been explored.

In this study, we investigated the antiangiogenic effect and the potential underlying mechanism of G-Rh2 in transgenic zebrafish embryos.

MATERIALS AND METHODS

Chemicals and reagents

We purchased 20(S)-G-Rh2 from Aladdin Co. Ltd., China. Dimethyl sulfoxide (DMSO) was purchased from Sigma Aldrich Chemicals. Pvt. Ltd., USA. G-Rh2 was dissolved in DMSO as 100 mM stock solution. The stock solution was stored at -20°C to maintain stability until use.

Trizol reagent was purchased from Invitrogen (Carlsbad, CA, USA). The PrimeScript RT reagent kit with gDNA Eraser was purchased from Takara (Dalian, China). Zebrafish VEGF antibody, mouse IgG horseradish peroxidase conjugated antibody and β-actin antibody were purchased from R&D systems (Minneapolis, MN, USA). The ECL advanced western blotting detection kit was purchased from Advansta (Menlo Park, USA). SU5416 was obtained from Sigma Aldrich (Shanghai, China).

Zebrafish maintenance and embryos collection

Wild-type (WT) zebrafish and transgenic zebrafish Tg (*fli1*:*EGFP*) expressing enhanced GFP in endothelial cells were provided by core facilities at the Zhejiang University School of Medicine. The two zebrafish strains were maintained as described in the Zebrafish Handbook (Westerfield, 2000). In brief, adult zebrafish were maintained in a controlled environment at 28.5°C with a 14 h:10 h light and dark cycle. Fish were fed three times a day with brine shrimp and occasionally with tropical fish food for 5 min. Zebrafish embryos were generated by natural pairwise mating and were raised at 28.5°C in embryonic culture medium water (0.75 mM $NaHCO_3$, 0.5 mM $MgSO_4 \cdot 7H_2O$, 2 mM $CaCl_2 \cdot 2H_2O$, and 0.08 mM KCl).

Drug administration and morphological observation

At 24 hours post-fertilization (hpf), WT embryos were dechorionated by treatment with protease 1 mg/mL for 5 min and washed using embryo water 5–6 times. Dechorionated embryos were arrayed in a 12-well plate, with approximately 20 embryos per well in 2 mL embryo water. Each well contained different concentrations of G-Rh2 (30, 42.43, 60, 84.85, 120 μmol) and plates were incubated for 48 h at 28.5°C. Three replicates were performed for every group. Embryos treated with 0.1% DMSO (solvent) served as a vehicle control. After drug exposure, the survival rate and morphological changes were examined at 12 h post-treatment (hpt), 24 hpt and 48 hpt. Images were captured using an Olympus Inverted Microscope (Olympus Corporation, Tokyo, Japan). Each experiment was repeated three times.

Assessment of vessel changes in zebrafish embryos by fluorescent microscopy

At 24 hpf, the Tg (*fli-1:EGFP*) zebrafish embryos were distributed in 12-well plates with 20 embryos per well. Each group was treated with various concentrations of G-Rh2 dissolved in embryo water for 24 h. The intersegmental blood vessels (ISVs) of embryos were observed and imaged at 24 hpt by fluorescent microscopy (Olympus Corporation). DMSO (0.1%) served as vehicle control and SU5416 (5 μmol) as positive control.

Quantitative real-time PCR (qPCR)

Zebrafish embryos at 24 hpf were treated with 0.1% DMSO and various concentration of G-Rh2 for 48 h. At 72 hpf, total RNA was isolated from samples of 30 zebrafish embryos per treatment group using Trizol reagent. RNA (1 μg) was reverse transcribed to cDNA using the PrimeScript RT reagent kit with gDNA Eraser according to the manufacturer's instructions. The quality and quantity of RNA were assessed by agarose gel electrophoresis and spectrophotometry. qPCR was performed using the TB Green Premix Ex Taq II (Tli RNaseH Plus) (TaKaRa) on the SteponePlus Real-Time PCR System (Thermo Fisher Scientific). Specific primers for *vegf*, *flt-1* and *kdr* mRNA (*vegf*-fw/rv, *flt1*-fw/rv, and *kdr*-fw/rv, respectively) were designed and the sequences are shown in Table I. *β-actin* was chosen as the reference gene for normalization of gene expressions. Standard reactions (20 μL) consisted of 10 μL 2×TB Green *Premix Ex Taq* II, 0.8 μL each primer (10 μM), 2 μL cDNA template (10 ng), 0.4 μL ROX Reference Dye (50×), and 6 μL sterile water. qPCR was performed as follows: an initial step at 95°C for 30 s, followed by 40 cycles at 95°C for 5 s and 60°C for 30 s. Each sample was amplified in triplicate. Data were analyzed by the $2^{-\Delta\Delta Ct}$ relative quantification method.

Western blot analysis

Zebrafish embryos at 24 hpf were treated with various concentrations of G-Rh2 and 0.1% DMSO as

control for 48 h. Embryos were then washed twice with PBS and lysed at 4°C in RIPA buffer for 30 min followed by centrifugation at 13200 × g for 20 min at 4°C. Protein concentration was quantified using the Bradford Protein Assay Kit (Beyotime, China) and equalized before loading. Equal amounts of total protein were mixed with sample buffer and heated to 100°C for 5 min. The proteins were separated by 12% SDS-PAGE and electrotransferred onto polyvinylidene fluoride membranes (Bio-Rad, USA). Blots were blocked with 5% skim milk in TBST for 3 h at room temperature and then incubated with zebrafish VEGF monoclonal antibody (1:800) at 4°C overnight. After washing, the membrane was incubated with Mouse IgG horseradish peroxidase conjugated antibody (*1:1000*) for 1 h at room temperature. Proteins bands were detected using an ECL advanced western blotting detection kit. β-actin was used as the loading control. Densitometry quantification was performed using Image J software *(*US National Institutes of Health; http://rsb.info.nih.gov/ij/*)*.

Table I. List of primers used for expression analysis.

Primer name	Sequence (5'–3')	Primer length
vegf-fw	TGCTCCTGCAAATTCACACAA	22
vegf-rv	ATCTTGGCTTTTCACATCTGCAA	24
flt1-fw	AACTCACAGACCAGTGAACAAGATC	25
flt1-rv	GCCCTGTAACGTGTGCACTAAA	22
kdr-fw	GCAGAATTCATTCCCATGCCGAACATTAC	29
kdr-rv	GTTAAGCTTAGTCTGAGGCGATCTTGAGG	29
ß-actin-fw	CGAGCAGGAGATGGGAACC	19
ß-actin-rv	CAACGGAAACGCTCATTGC	19

Statistical analysis

Expression analysis and western blot experiments were repeated three times. Data were analyzed using GraphPadPrism 5.0 software (GraphPad Software Inc., San Diego, CA, USA). Statistical significance was assessed one-way analysis of variance and a probability value of $P < 0.05$ was considered significant.

RESULTS

Morphological observations

Zebrafish embryos at 24 hpf were dechorionated by protease prior to drug treatment (Fig. 1). To determine the optimum drug concentration, embryos were exposed to various concentrations of G-Rh2 ranging from 30–120 μM and morphological changes of the G-Rh2-treated and control embryos at 12, 24 and 48 hpt were examined. The embryos treated with G-Rh2 up to 84.85 μM did not show any morphological defects compared with controls, but G-Rh2 at 120 μM caused morphological changes such as tail bending and pericardial edema, as shown in Figure 2. Therefore, we selected 84.85 μM as our maximum treatment concentration in subsequent experiments.

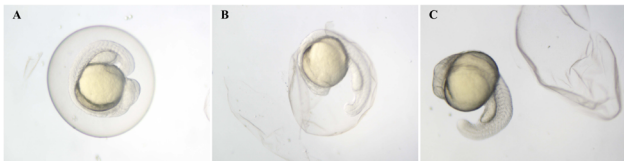

Fig. 1. Dechorionation of zebrafish embryos by protease (1 mg/ml) at 24 hpf. Embryos in the chorion. B. Embryos separating from chorion. C. Embryos out of the chorion. hpf: hours post-fertilization.

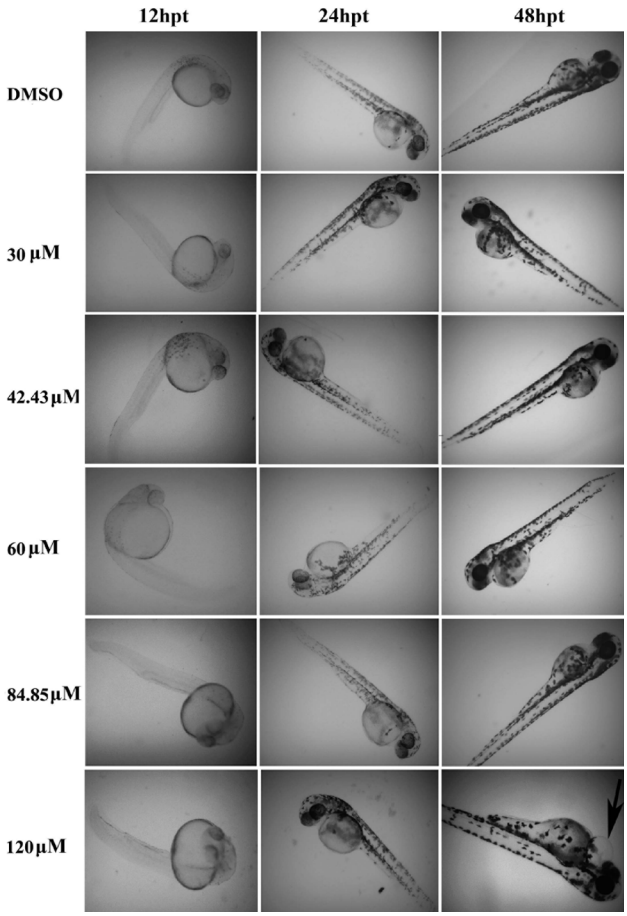

Fig. 2. Morphological changes of zebrafish embryos after G-Rh2 treatment. Zebrafish embryos at 24 hpf were treated with 0.1% DMSO and various concentrations of G-Rh2 (30, 42.43, 60, 84.85, 120 μmol) for 48 h. The arrow denotes pericardial edema after treatment with high concentration of G-Rh2 at 120 μmol. hpt: hours post-treatment.

Anti-angiogenic activity of G-Rh2 in zebrafish

Tg (*fli-1:EGFP*) transgenic zebrafish embryos at 24 hpf were used to evaluate the potential anti-angiogenic activity of G-Rh2. Embryos (24 hpf) were treated with SU5416 (positive control, 5 μM), 0.1% DMSO (vehicle control), or G-Rh2 (30, 42.43, 60 and 84.85 μM) for 24 h and then examined at 48 hpf by fluorescence microscopy. As shown in Figure 3, ISV angiogenesis was most easily observed in the embryos at 48 hpf. Treatment with 0.1% DMSO had no effect on ISV formation (Fig. 3A and A′). SU5416 treatment showed remarkable suppression of ISV formation (Fig. 3B and B′). G-Rh2 inhibited ISV development in zebrafish embryos in a dose-dependent manner compared with the control. The anti-angiogenic effect of G-Rh2 was seen at concentrations ranging from 42.43 to 84.85 μM (Fig. 3D–F and D′–F′). At the concentration of 84.85 μM G-Rh2, ISV development was almost completely suppressed (Fig. 3F and F′).

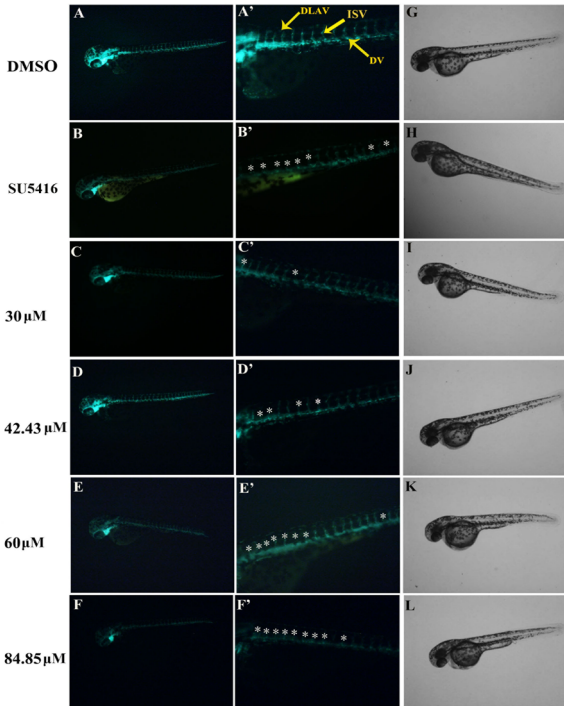

Fig. 3. Anti-angiogenic effect of G-Rh2 on *Tg* (*Fli1-EGFP*) zebrafish embryos. 24 hpf embryos were treated for 12 h with (A) 0.1% DMSO (vehicle control); (B) 5 μM SU5416 (positive control); (C) 30 μM G-Rh2; (D) 42.43 μM G-Rh2; (E) 60 μM G-Rh2; or (F) 84.85 μM G-Rh2. Magnified views of A–F are shown in A′–F′. Morphological observation of zebrafish embryos is shown in G–L. The dorsal longitudinal anastomotic vessel (DLAV), dorsal aorta (DA) and intersegmental vessels (ISVs) of zebrafish embryos are indicated by the yellow arrows. Defects in ISV formation are indicated by the white asterisk.

qPCR

The VEGF signaling pathway plays an important role in angiogenesis and is well studied in zebrafish vascular development (Li *et al*., 2012; He *et al*., 2014; Karthik *et al*., 2014). Vegf is the most studied factor in zebrafish angiogenesis. VEGF receptors are highly specific for VEGF and are markedly upregulated during periods of tumor growth (Cross *et al*., 2003). To determine the molecular mechanism of G-Rh2-mediated anti-angiogenesis in zebrafish, we examined the gene expressions of *vegf* and its receptor VEGFR-1 (*flt-1*) and VEGFR-2 (*kdr*) as representatives of the VEGF signaling pathway. As shown in Figure 4, G-Rh2 treatment resulted in significantly decreased *vegf* mRNA expression in a dose-dependent manner compared with controls (Fig. 4A). However, the expression levels of *flt1* and *kdr* mRNA were not significantly altered by G-Rh2 treatment (Fig. 4B–C).

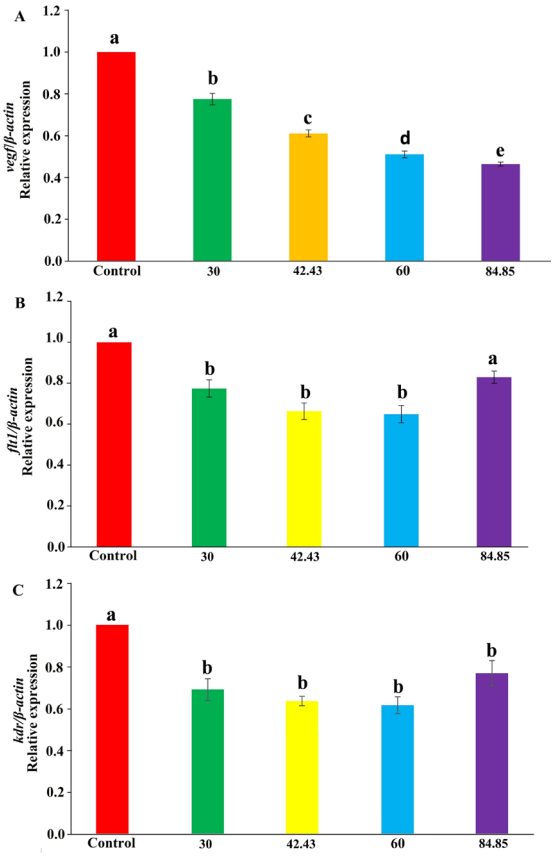

Fig. 4. qPCR analysis of VEGF signaling pathway genes in zebrafish embryos treated with G-Rh2. qPCR analysis of *vegf* (A), *flt1* (B) and *kdr* (C) mRNA expression in 72 hpf zebrafish embryos treated with different concentrations of G-Rh2 or 0.1% DMSO (vehicle control). Data are shown as mean ± SEM of triplicate experiments. Different letters (a–e) above the columns indicate significant differences ($P < 0.05$).

Western blot analysis

We further analyzed Vegf protein expression in G-Rh2-treated and control zebrafish embryos by western blot analyses. The results showed that Vegf protein expression level was substantially decreased in response to G-Rh2 treatment compared with the control (Fig. 5). These data suggested that G-Rh2 may inhibit angiogenesis by downregulation of Vegf expression in zebrafish embryos.

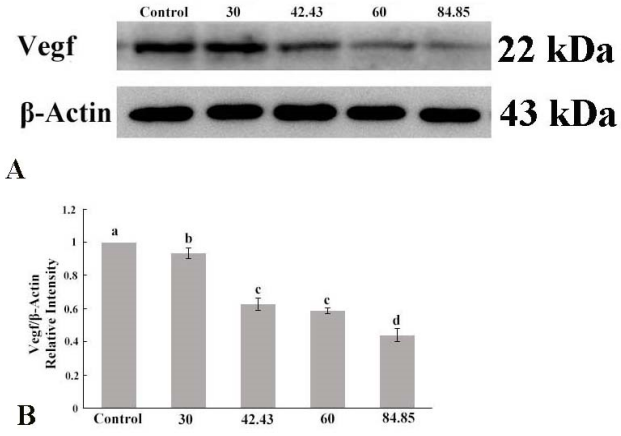

Fig. 5. Western blot analysis of VEGF protein expression in G-Rh2 treated embryos. Western blot of Vegf expression in 72 hpf zebrafish embryos treated with different concentrations of G-Rh2 or 0.1% DMSO (vehicle control). β-Actin was used as loading control. Different letters (a–d) above the columns indicate significant differences ($P < 0.05$).

DISCUSSION

Angiogenesis plays an important role in tumor development, progression, and metastasis (Mantovani, 2010), and antiangiogenic cancer therapy strategies have been extensively studied (Folkman, 1971; Jansen *et al*., 2004). G-Rh2 is natural glycoside extracted from the traditional medicinal plant red ginseng. Previous *in vitro* studies indicated that G-Rh2 shows low toxicity, with only a few side effects, and G-Rh2 has been considered as a nutrient with anticancer properties (Zeng and Tu, 2003). G-Rh2 exhibits cytotoxic and apoptotic effects in hepatoma and leukemia Reh cells (Park *et al*., 2012; Xia *et al*., 2014). G-Rh2 was also shown to inhibit the tumor cell proliferation and invasiveness in glioma and prostatic cancer (Wu *et al*., 2011; Zhang *et al*., 2015; Li *et al*., 2018) and exhibit anti-cancer activity in human colorectal cancer cells (Han *et al*., 2016). In addition, G-Rh2 displayed a suppressive effect on tumor growth in nude mice bearing tumors derived from human ovarian cancer cells (Nakata *et al*., 1998). G-Rh2 also showed antiangiogenesis in a Lewis pulmonary carcinoma mouse model (Cui and Qu, 2011). Therefore, study of the antitumor mechanism of G-Rh2 has become a hotspot in anti-cancer traditional Chinese medicine research. Here we explored the antiangiogenic activity and molecular mechanism of G-Rh2 in zebrafish embryos.

Zebrafish embryos have been widely used as a live *in vivo* vertebrate model for studying inhibitors of angiogenesis. Zebrafish embryos are especially suitable for vascular development analysis because of their small size, transparency, ease of manipulation and rapid development (Santoro, 2014). Moreover, zebrafish embryos can survive for a week without a blood circulation or vascular system (Li *et al*., 2016). Many anti-angiogenic drugs tested in this model demonstrated similar responses to those observed in mammalian systems (Cross *et al*., 2003; Langheinrich, 2003). Angiogenesis is a critical process involved in tumor growth, development and metastasis. The formation of ISVs in zebrafish embryos is a paradigm for studying angiogenesis *in vivo* (Karthik *et al*., 2014). Transgenic zebrafish expressing EGFP in blood vessels allow for observation of angiogenesis in live embryos under a fluorescence microscope (Tran *et al*., 2007). We used the Tg (*fli1-EGFP*) transgenic zebrafish, which express EGFP in endothelial cells, to study anti-angiogenic activity of G-Rh2. We treated zebrafish embryos with G-Rh2 at 24 hpf when the sprouting of ISVs from the vasculogenic vessel and posterior cardinal vein initiated (Letamendia *et al*., 2012). We did not observe any morphological changes in embryos treated with G-Rh2 up to 84.85 μM but higher concentrations at 120 μM caused tail bending and pericardial edema at 72 hpf. Moreover, the antiangiogenic effect of G-Rh2 was observed from 42.43 to 84.85 μM, with a significantly reduced number of ISVs with increasing concentration of the drug. These results indicate that the optimal concentration of G-Rh2 was 84.85 μM, while concentrations of 120 μM or above were toxic to the zebrafish embryos.

VEGF signaling plays a key role in the process of angiogenesis (Ferrara *et al*., 2003). VEGF is currently the mostly studied growth factor in zebrafish angiogenesis. (Paramasivam *et al*., 2012). Paramasivam *et al*. (2012) showed that thymoquinone has potent antiangiogenic activity by downregulating VEGF in the zebrafish model. Karthik *et al*. (2014) reported that syringic acid can inhibit the formation of blood vessels by downregulation of VEGF-mediated angiogenesis in zebrafish embryos. Currently, three VEGF receptors have been found in zebrafish, namely VEGFR1, VEGFR2 and VEGFR3 (Bussmann *et al*., 2008). He *et al*. (2014) found that auranofin suppressed VEGF signaling pathway genes (*vegf*, *flt1*, *kdr*) but had no impact on thioredoxin reductase in zebrafish. In our study,

we selected VEGFR-1 (*flt1*) and VEGFR-2 (*kdr*), as these two receptors are expressed mainly in vascular endothelial cells (Goishi and Klagsbrun, 2004). qPCR showed that G-Rh2 only downregulated *vegf* mRNA levels but did not impact gene expression of its receptors (*flt1, kdr*) in zebrafish. We further examined the protein expression of Vegf in zebrafish embryos using western blot analysis and found that G-Rh2 also reduced Vegf expression in zebrafish embryos in a dose-dependent manner. Together these results demonstrate that G-RH2 downregulates Vegf expression in the zebrafish model.

In conclusion, our study suggested for the first time that G-Rh2 inhibits blood vessel formation in zebrafish. We have also shown that the molecular mechanism of the anti-angiogenic activity of G-Rh2 may be due to inhibition of Vegf in zebrafish. This study provides a theoretical basis for further elucidation of the anti-tumor mechanism of G-Rh2 and demonstrated the applicability and feasibility of screening the antiangiogenic activity of natural compounds in the zebrafish model.

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Statement of conflict of interest

The authors have declared no conflict of interest.

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