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

MA Liman1, QI Yongxiao1, Wang Wenji* and Zhong Qianyi*2

1School of Medicine, Taizhou University, Taizhou, Zhejiang 318000, P.R. China.
2School of Life Science, Taizhou University, Taizhou, Zhejiang 318000, P.R. China.
*Corresponding author: wangwj@tzc.edu.cn; zhongqy@tzzxyy.com

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(β1-EGFP) transgenic zebrafish with fluorescent blood vessels were studied to evaluate vascular development defects. G-Rh2 treatment up to 90 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 90 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.

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, 2002; Han et al., 2017). The advantages of studying angiogenesis in zebrafish include 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 (fli-1: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 NaHCO₃, 0.5 mM MgSO₄·7H₂O, 2 mM CaCl₂·2H₂O, 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 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 PB 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-ΔΔCt 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.

<table>
<thead>
<tr>
<th>Primer name</th>
<th>Sequence (5′–3′)</th>
<th>Primer length</th>
</tr>
</thead>
<tbody>
<tr>
<td>vegf-fw</td>
<td>TGCTCTTGCAAAATTCACACA</td>
<td>22</td>
</tr>
<tr>
<td>vegf-rv</td>
<td>ATCTTGGCTTTTGACATCTGCA</td>
<td>24</td>
</tr>
<tr>
<td>flt1-fw</td>
<td>AACTCACAGACCACTGAAACGAT</td>
<td>25</td>
</tr>
<tr>
<td>flt1-rv</td>
<td>GCCCTGAAACGCTGCACCTAAA</td>
<td>22</td>
</tr>
<tr>
<td>kdr-fw</td>
<td>GCAGAATTCCATCATCATGGCAGAATGACATTAC</td>
<td>29</td>
</tr>
<tr>
<td>kdr-rv</td>
<td>GTTAAGCTTAGTCTGAGGCGATCTTGAGG</td>
<td>29</td>
</tr>
<tr>
<td>β-actin-fw</td>
<td>CGACGGAGAATGCGAAGAC</td>
<td>19</td>
</tr>
<tr>
<td>β-actin-rv</td>
<td>CAACGGAAACGCTGCATGCG</td>
<td>19</td>
</tr>
</tbody>
</table>

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 (fl1-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’).

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 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).
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.

![Western blot analysis](image)

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, 1996; Jansen et al., 2004). G-Rh2 is a 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., 2003; Xia et al., 2014). G-Rh2 was also shown to inhibit the tumor cell proliferation and invasiveness in glioma and prostatic carcinoma (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 EGF 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 EGF 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 downregulating 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.

ACKNOWLEDGMENTS

Work was supported by the Experimental Animal Science and Technology Project of Zhejiang Province (grant numbers 2017C37154) and Taizhou Science and Technology Plan Project (grant numbers 15ny11). We thank Edanz Group (www.edanzediting.com/ac) for editing a draft of this manuscript.

Statement of conflict of interest

The authors have declared no conflict of interest.

REFERENCES


Jin, X., Che, D.B., Zhang, Z.H., Yan, H.M., Jia, Z.Y.,


