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Pro-angiogenic activity of isoliquiritin on HUVECs *in vitro* and zebrafish *in vivo* through Raf/MEK signaling pathway

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ABSTRACT

Aims: Liquorice is a widely used herbal medicine for treating various diseases native to southern Europe and parts of Asia. Isoliquiritin (ISL), a licorice root-derived flavonoid, has been reported to exhibit antioxidant, anti-inflammatory, anti-genotoxic activity and anti-depression activities. This study was aimed to explore the pro-angiogenic activity of ISL and explicate the underlying mechanism.

Main methods: In vitro, ISL-treated human umbilical vein endothelial cells (HUVECs) were analyzed for cell viability, cell migration and tube formation. *In vivo*, pro-angiogenic effects were evaluated for the intersegmental vessels (ISVs) formation in transgenic zebrafish embryos [Tg(*fli-1*: EGFP)]. Furthermore, a blocking assay with eight pathways-specific kinase inhibitors were also used to determine the potential pro-angiogenic mechanism of ISL.

Key findings: ISL counteracted tyrosine kinase inhibitor II (VRI)-induced endothelial cell apoptosis and promoted cell migration and tube formation in HUVECs. ISL markedly rescued ISVs loss induced by VRI in zebrafish embryos, probably by activating vascular endothelial growth factor receptor-2 (VEGFR-2), phosphoinositide 3-kinase (PI3K), Raf and mitogen-activated protein kinase (MEK)-dependent signaling pathways.

Significance: Our study first discovered and confirmed the pro-angiogenic activity of ISL both in HUVECs and zebrafish. Thus, ISL could be developed as a potential therapeutic agent by the role of pro-angiogenic activity for the treatment of cardiovascular diseases, cerebrovascular diseases and other vascular diseases.

1. Introduction

Angiogenesis, the branching of new vascular sprouts out of pre-existing blood vessels, has an essential role in development, reproduction and repair [1]. Disturbance of angiogenic balance can lead to lots of pathological diseases, including extensive angiogenesis (*e.g.* rheumatoid arthritis, psoriasis, blinding eye disease and cancer growth and metastasis) and insufficient angiogenesis (*e.g.* atherosclerosis, stroke, myocardial infarction and chronic wounds) [2]. In recent years, angiogenesis has emerged as a new therapeutic strategy for tumor and stroke treatment. On the one hand, angiogenesis contributes to the normalization of tumor vascular structure, which could restore proper tumor perfusion and oxygenation, limiting tumor cell invasiveness and improving the effectiveness of anticancer treatments [3]. On the other hand, angiogenesis could enhance cerebral blood flow including increasing collateral recruitment through inducing a number of pro-angiogenic factors and circulating endothelial progenitor cells (EPCs), which has become a more effective therapy for ischemic stroke [4]. Thus, it is reasonable and beneficial to explore and screen pro-angiogenic agent for vascular-related diseases treatment.

Zebrafish has become a prominent vertebrate model for studying embryonic development, modeling human diseases, screening for pharmaceutical drugs, and dissecting biological pathways [5]. 71% of human proteins (and 82% of disease-causing human proteins) have an obvious orthologue in zebrafish [6]. Zebrafish provides a series of advantages such as rapid development, optical transparency, large numbers of offspring. Moreover, the zebrafish embryos exhibit a transparent cardiovascular system during early development, allowing direct observation of blood flow and the development of the system's related organs, without the need for complex instrumentation [7]. In recent years, more and more phenotype-based anti- or pro-angiogenic drugs have been successfully discovered by using vasculature-specific labeled

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transgenic zebrafish model [8–11].

Liquorice is the root of *Glycyrrhiza uralensis* Fisch. or *Glycyrrhiza glabra* L., Leguminosae. It is a widely used herbal medicine for treating various diseases native to southern Europe and parts of Asia [12]. To our interest, liquorice was reported to have anti-apoptotic and neuroprotective effect on the treatment of ischemia-induced brain damage [13]. *Glycyrrhiza glabra* was also reported to have a cardioprotective effect against ischemia-reperfusion injury (I-R) in rats [14]. Ankaferd Blood Stopper (ABS), a unique traditional herbal mixture containing *Glycyrrhiza glabra*, benefits for wound healing and contributes to restore and maintain tissue homeostasis in a variety of diseases [15]. Isoliquiritin (ISL), a water soluble component of licorice root, has been reported to possess a broad spectrum of pharmacological activities including antioxidant, anti-inflammatory, anti-genotoxic activity and anti-depression activities [16–18]. We speculated that ISL may have an extra pro-angiogenic effect on the therapy of pathological disease caused by insufficient angiogenesis. Thus, the aim of our study was to explore and confirm the pharmacological activity in angiogenesis of ISL and explicate its underlying mechanism through human umbilical vein endothelial cells (HUVECs) and transgenic zebrafish.

2. Materials and methods

2.1. Cell culture and chemicals

HUVECs were obtained from Lonza (Walkersville, MD, USA). HUVECs were cultured in endothelial growth medium-2 (EGM-2, Walkersville, MD, USA) or endothelial basal medium-2 (EBM-2, Walkersville, MD, USA) according to the assay condition. HUVECs at early passage (3–7 passages) were used in all experiments. Cells were incubated at 37 °C in 5% CO_2 (v/v). Dimethyl sulfoxide (DMSO) and 1-phenyl 2-thiourea (PTU) were purchased from Sigma-Aldrich (St. Louis, MO, USA). ISL (CAS no. 5041-81-6, purity ≥ 98%) was purchased from Chengdu Pufei De Bio Co. (Chengdu, China), its chemical structure was shown in Fig. 1. Basic fibroblast growth factor (bFGF) was purchased from R&D system (Minneapolis, USA). VEGFR tyrosine kinase inhibitor II (VRI), p38 mitogen-activated protein kinase (p38 MAPK) inhibitor (P38i), extracellular regulated kinase 1/2 (ERK1/2) inhibitor (GDC-0994), Src inhibitor (PP2), protein kinase B (Akt) inhibitor (MK2206) were purchased from ApexBio (Houston, TX, USA). VEGFR2 inhibitor (Apatinib), Raf kinase inhibitor (Sorafenib), Phosphoinositide 3-kinase (PI3K) inhibitor (LY294002), mitogen-activated protein kinase kinase (MEK) inhibitor (PD0325901), Recombinant Human $VEGF_{165}$ were purchased form Beyotime Biotechnology (Shanghai, China).

2.2. Maintenance of zebrafish

Transgenic zebrafish (*fli-1*: EGFP) expressing enhanced green fluorescent protein (EGFP) in the endothelial cells were obtained from Model Animal Research Center of Nanjing University. Adult zebrafish were maintained at 28.5 °C and pH of 7 ± 0.2 in 14:10 h (h) light/dark photoperiod [19,20].

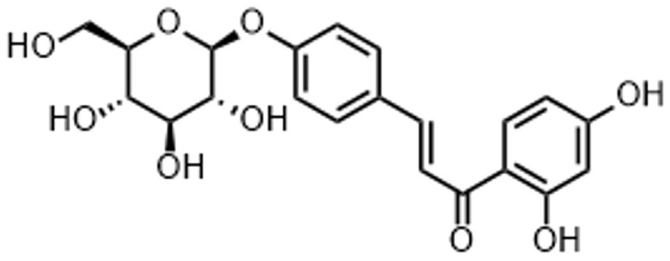


Fig. 1. Chemical structure of isoliquiritin (ISL).

2.3. Embryos collection and drug treatment

Zebrafish embryos were generated by natural pairwise mating and raised at 28.5 °C in embryo water (0.2 mg/ml of Instant Oceans Salt in distilled water). Healthy embryos were dechorionated with 1 mg/ml of pronase (Sigma-Aldrich, MO, USA) immediately prior to 24 hour post-fertilization (hpf) and distributed into a 24-well microplates for treatment and fluorescence observation (20 embryos per well, 3 replicates).

2.3.1. VRI-treated vascular insufficient model

Embryos at 24 hpf were pre-treated with 575 nM VRI for 4 h. At 28 hpf, the VRI was washed out and replaced with either 0.1% DMSO (v/v) embryo medium or four concentrations of ISL (50, 100, 200, 500 μM) for another 20 h. Embryos without any treatment served as control, embryos pre-treated with VRI and then 0.1% DMSO served as negative control.

2.3.2. Specific inhibitors assay in VRI-treated zebrafish

Embryos at 24 hpf were pre-treated with 575 nM VRI for 4 h. At 28 hpf, the VRI was washed out and replaced with either 0.1% DMSO (v/v) embryo medium or ISL (500 μM) only and ISL (500 μM) co-treatment with inhibitors for another 20 h.

All zebrafish studies were approved by the Institutional Animal Care and Use Committee at Nanjing Tech University.

2.4. In vitro cell viability assay

HUVEC (4000/well) were seeded in 96-well plate with EGM-2 medium (100 μl per well) for 24 h for attachment. Then cells were treated in EBM-2 for 48 h (150 μl per well) with VEGF (50 ng/ml) for stimulating growth completely and VRI (200 nM) for inhibiting growth or inducing apoptosis partly. Cells in EBM-2 medium served as blank. Cells receiving VEGF (50 ng/ml) only and VEGF (50 ng/ml) plus VRI (200 nM) served as control and negative control, respectively. Six concentrations of (5, 10, 30, 60, 100, 500 μM) ISL plus VEGF (50 ng/ml) and VRI (200 nM) were used as ISL treatments groups. After incubation for 48 h, cell growth was assessed by a cell-counting kit-8 (CCK-8, Dojindo, Japan) according to the protocol provided. The spectrophotometric absorbance of each well was measured by a multi-detection microplate reader (Synergy HT, BioTek, USA) at a wavelength of 450 nm. Each treatment was performed in 6 independent experiments. The percentage of cell viability was expressed using blank wells at 100%.

2.5. In vitro wound healing assay

HUVECs in EGM-2 were seeded into 24-well plates precoated with 0.1% Gelatin (Sigma-Aldrich, St. Louis, USA), and grown overnight to achieve a fusion rate of approximately 80%. The monolayer cells were wounded by scratching with P200 pipette tips and washed with PBS to remove the non-adherent and damaged cells. EBM-2 containing the indicated concentrations of ISL (10, 30, 60 μM) was then added into the wells. Cells receiving 0.1% DMSO only and bFGF (30 ng/ml) served as vehicle control and positive control, respectively. After continuous cultivation for 10 h, cells were washed by PBS and fixed with 0.1% crystal violet methanol staining solution (Merck, Germany) for 10 min. Then the staining solution was washed by ddH2O. Images were taken by an inverted microscope (IX71, Olympus, Japan). The migrated cells were counted manually. The values were observed from four randomly selected fields. The percentage of migration was expressed using control wells at 100%.

2.6. In vitro tube formation assay

Matrigel (growth factor reduced; BD Biosciences, USA) was thawed slowly at 4 °C overnight. Each well of pre-chilled 96-well plates was

coated with 50 μl of matrigel, incubated and solidified at 37 °C for 45 min. HUVECs at the density of 2×10^4 cells per well (200 μl) in EBM-2 containing the indicated concentrations of ISL (10, 30, 60 μM), 0.1% DMSO and bFGF (30 ng/ml) were placed onto the matrigel layer. After incubation for 16 h, HUVECs were fluorescently labeled with CM-DiI (Invitrogen, CA, USA) for 15 min according to the manufacturer's instructions. Images were captured in red fluorescent field at 100× magnification using an inverted microscope (IX71, Olympus, Japan) from four different fields in each well. The tube length was quantified by Image Pro Plus software. The percentage of tube length was expressed using control wells at 100%.

2.7. Zebrafish intersegmental blood vessels (ISVs) formation assay

At 48 hpf, the embryos were removed from microplates and observed for ISVs under a fluorescence microscope (IX71, Olympus, Japan). We randomly chose 20 embryos from each group for photographing and quantitative analysis. The length of ISVs was quantified with Image-Pro Plus software. Drug effect was calculated by the percentage of ISVs length and was expressed using control wells at 100%.

2.8. Statistical analysis

All statistical analyses were expressed as mean ± SEM using GraphPad Prism 5.0. Statistical significance was analyzed using one-way ANOVA followed by Dunnett's test or Tukey's test. P values < 0.05 were considered significant. Each experiment was done in triplicate and repeated for three times at least.

3. Results

3.1. ISL counteracted VRI-induced endothelial cell apoptosis of HUVECs

In order to evaluate the pro-proliferative capacity of ISL, we investigated the effects of VRI-induced cell apoptosis and co-treatment with ISL in HUVECs after stimulation with VEGF (50 ng/ml). Fig. 2 showed that in HUVECs, VRI (200 nM) treatment decreased cell viability, induced apoptosis up to 48% of control group (VEGF treatment at 50 ng/ml). The co-treatment of VRI and ISL (5, 10, 30, 60, 100, 500 μM) was able to counteract the cells from apoptosis, as well as promote the proliferation of HUVECs (from 22% to 85% of the negative control group). ISL could effectively reverse the apoptosis and cytotoxicity brought from VRI (200 nM) at 10 μM or higher concentrations. Even at 500 μM, ISL didn't obstruct the proliferative capacity of cells and exhibit no extra cytotoxicity. In all, the data showed that ISL possessed endothelial protective effect (cell survival) in addition to pro-angiogenic property (cell proliferation).

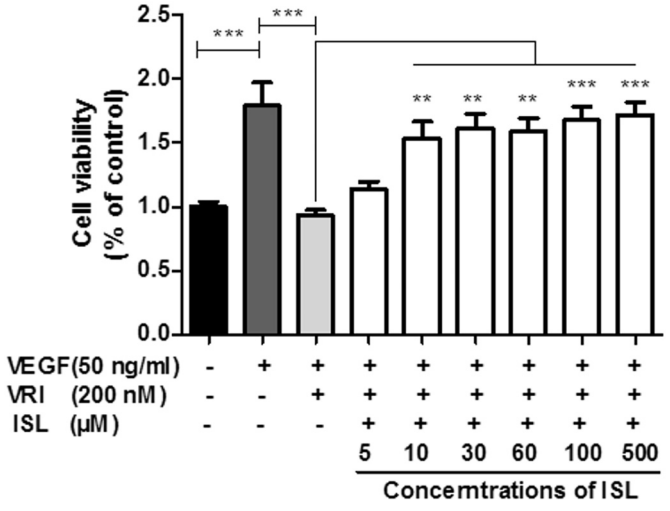


Fig. 2. ISL recused VRI-induced apoptosis and promoted the proliferation of HUVECs. HUVECs (4000 cells/well in EGM-2) were plated in 96-well culture plates. After 24 h, the medium was changed to fresh EBM-2 containing VEGF (50 ng/ml), 200 nM VRI or co-treatment with various concentrations of ISL (5–500 μM) as indicated. After 48 h of treatment, cell viability was measured by CCK-8 assay. Each point represents mean ± SEM ($n = 6$) from 6 independent experiments. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$. The data were analyzed by one-way ANOVA followed by the Tukey's test for comparisons of all treated groups with control group.

3.2. ISL promoted the wound healing of HUVECs

Cell migration is one of the hallmarks of angiogenesis and occurs in the earlier steps of the angiogenic cascade. Wound healing assay was performed to investigate the pro-migratory effect of ISL. As Fig. 3A shown, bFGF (30 ng/ml) extremely narrowed the wound sizes of HUVECs. Treatment with ISL 10, 30 and 60 μM also promoted the wound healing of HUVECs between the scratch on both sides. Moreover, at concentrations of 30 μM and 60 μM, the pro-migration ability of ISL was increased to 146% and 151% of the control group, almost close to the positive control group (bFGF, 156% of control) in Fig. 3B. Taken together, ISL could promote the wound healing migration process of HUVECs.

3.3. ISL promoted the tube formation of HUVECs

Tube formation assay was carried out to test the effect of ISL on the formation of chord-like networks in HUVECs. The capillary-like tubes were observed after 12 h of incubation with ISL. As shown in Fig. 4A, bFGF (30 ng/ml) dramatically promoted capillary tube formation. Similarly, ISL at 10, 30 and 60 μM significantly promoted network formation to 138%, 166% and 188% compared with control group at 12 h post seeding, respectively (Fig. 4B). Therefore, ISL could promote HUVEC network formation in a dose-dependent manner.

3.4. ISL restored the VRI-induced ISVs insufficiency in zebrafish embryos

VRI is a small molecule that strongly inhibits the kinase activity of both VEGF receptor 1 and 2 [21]. When 575 nM VRI was added at 24 hpf and washed at 28 hpf, the development of ISVs of zebrafish embryos were significantly inhibited until 48 hpf (Fig. 5I, B and B′). During this time period, ISL was added to determine if it could cause more ISV recovery, an indicator of angiogenesis, compared to the negative control group. Therefore, we chose this pre-inhibited vascular structure by VRI to evaluate pro-angiogenic activity of ISL in zebrafish embryos. As shown in Fig. 5, the growth of ISVs was strongly inhibited almost 53% following treatment with 575 nM VRI for 4 h. ISL rescued the VRI-induced ISVs insufficiency and promoted the recovery of ISV in a dose-dependent manner from 50 μM to 500 μM after incubation for 20 h. Meanwhile, ISL could recover almost 82% of ISVs formation at the concentration of 500 μM without observing abnormal phenotype similar to teratogenic toxicity (Supplementary Fig. 1). Thus, we confirmed the pro-angionic activity of ISL on VRI-induced ISVs insufficiency in zebrafish embryos and chose the concentration of ISL at 500 μM for the following mechanism study.

3.5. The pro-angiogenic activity of ISL was blocked by the inhibitors of downstream signaling targets of VEGFR2 in VRI-treated zebrafish embryos

In order to explore the possible targets underling the pro-angiogenic activity of ISL, we chose eight specific small molecular inhibitors related to VEGF signaling pathways and investigated the exact role of VEGFR2, Src, PI3K, Akt, p38 MAPK, Raf, MEK, ERK1/2 on ISL-induced angiogenesis through zebrafish model (Table 1). VEGFR2, PI3K, Raf, MEK had been reported to be crucial downstream signaling targets that

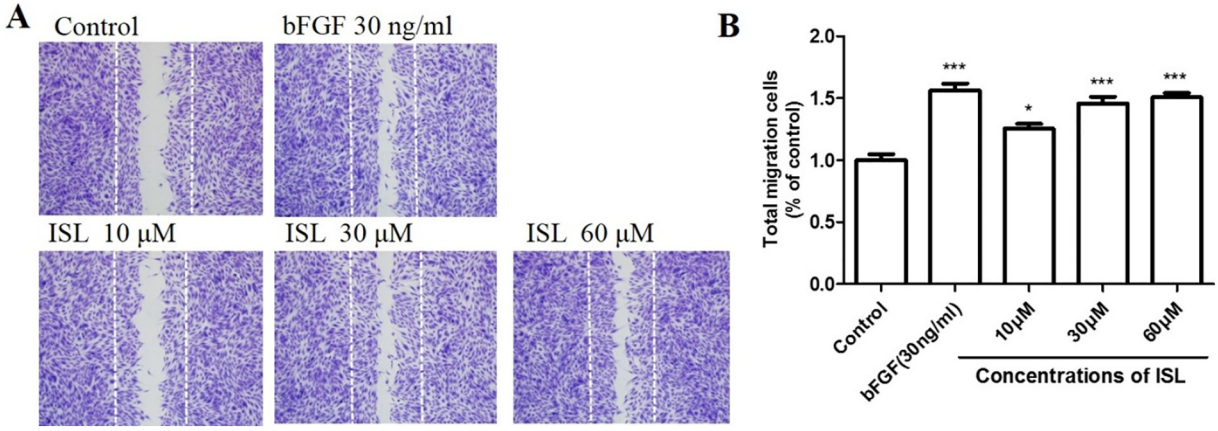


Fig. 3. ISL promoted HUVECs migration. (A) Confluent monolayer of HUVECs was wounded (scratched by pipette tips, the wound was shown between the two white dash lines in each photo) and then was treated with 0.1% DMSO (control), 30 ng/ml bFGF (positive control) and various concentrations of ISL (10–60 μM) for 10 h. Cells were stained with Gimsa solution and photoed under inverted microscope. (B) Quantification of the number of migrated cells (cells between the two white dash lines) after 10 h exposure. Each point represents mean ± SEM ($n = 4$) from 3 independent experiments. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$. The data were analyzed by one-way ANOVA followed by the Dunnett's test for comparisons of all treated groups with control group.

are involved in VEGF-induced angiogenesis [22,23]. VEGFR2 is essential for the function of vascular endothelial and can induce cellular processes including cell migration, survival and proliferation [23]. Apatinib, a highly selective VEGFR2 inhibitor and has been used to treat cancer by inhibiting tumor growth and angiogenesis [24]. As a main downstream protein kinase of VEGFR2, PI3K activation could contribute to rescue both hematopoietic and angiogenic defects in zebrafish. LY294002, a specific PI3K inhibitor, which acts on the ATP (adenosine 5′-triphosphate) binding site of PI3K [25]. The PI3K-dependent pathway is regulated by a wide-range of upstream signaling proteins and it also regulates many downstream effectors by collaborating with various compensatory signaling pathways, primarily with Raf/MEK pathway [26]. The inhibitors of Raf and MEK we used are sorafenib and PD0325901, respectively.

Our results showed that ISL at 500 μM rescued the growth of ISVs by 42% compared with VRI-treated group. However, the co-treatment of ISL and these inhibitors decreased by 38%–46% (exhibit no recovery of ISVs in Fig. 6E, G, I, K) compared with ISL-treated only group. While treatment with 0.5 μM apatinib (VEGFR2 inhibitor), 5 μM LY294002 (PI3K inhibitor), 0.25 μM sorafenib (Raf inhibitor) and 0.25 μM PD0325901 (MEK inhibitor) only did not affect the normal blood vessel development in zebrafish (Fig. 6D, F, H, J), neither the gross morphology of the developing embryos (Supplementary Fig. 1). Specifically, our results showed that VEGFR2, PI3K, Raf, MEK inhibitor could reverse vascular restorative effect of ISL on VRI-treated blood vessel loss zebrafish model, which means the pro-angiogenic activities of ISL were blocked by co-treatment with these inhibitors. While Src, p38 MAPK, Akt, and ERK1/2 inhibitors all failed to abolish the vascular restorative effect of ISL (data not shown). The above results suggested that VEGFR2, PI3K, Raf, MEK, but not Src, p38 MAPK, Akt, ERK1/2 were probably involved the pro-angiogenesis effect of ISL.

4. Discussion

In this study, we were able to demonstrate that the pro-angiogenic activity of ISL in both *in vitro* HUVECs and *in vivo* zebrafish assays. The results showed that ISL could rescue and enhance proliferation, migration and tube formation in HUVECs *in vitro*, which was consistent to the results showed in VRI-treated zebrafish embryos *in vivo*. All of these results suggested that ISL possesses pro-angiogenic activity.

Angiogenesis insufficiency is associated with various human diseases (*e.g.* atherosclerosis, stroke, myocardial infarction and chronic wounds). During zebrafish embryonic development, formation of normal blood vessels is essential for tissue generation. VRI-induced vascular insufficiency in zebrafish could mimic the disruption of vascular regeneration in pathological conditions during anti-angiogenic

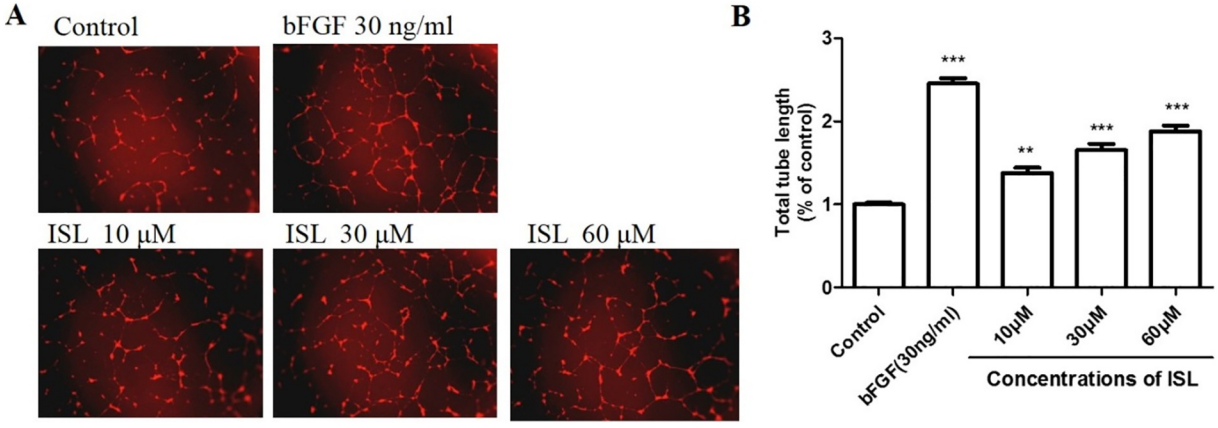


Fig. 4. ISL promoted the tube formation of HUVECs. (A) HUVECs was treated with 0.1% DMSO (control), 30 ng/ml bFGF (positive control) and various concentrations of ISL (10–60 μM) for 12 h. The capillary-like tubes were photoed under inverted microscope. (B) The vessel length in each treatment conditions were quantified by Image-Pro Plus. Each point represents mean ± SEM ($n = 4$) from a representative experiment. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$. The data were analyzed by one-way ANOVA followed by the Dunnett's test for comparisons of all treated groups with control group.

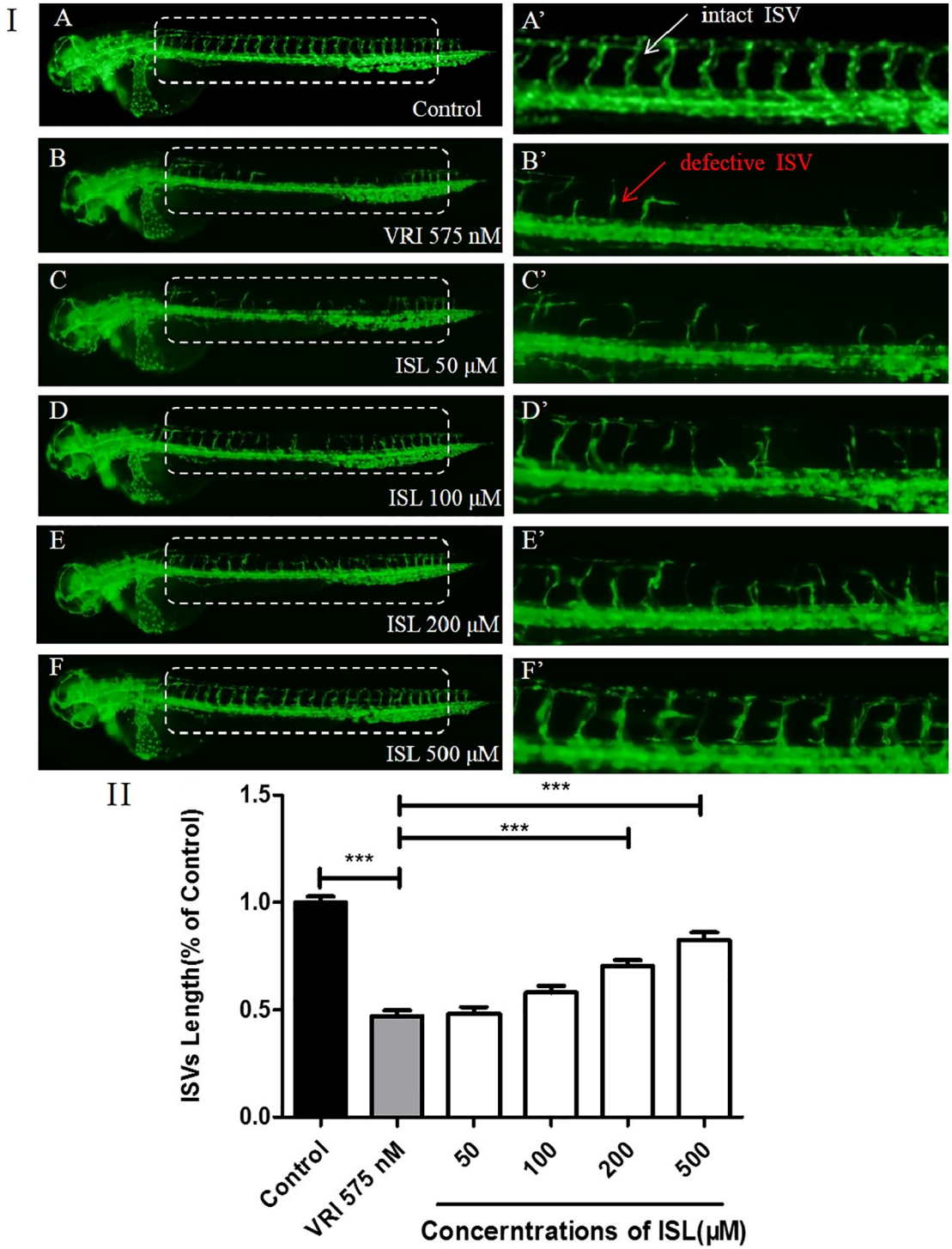


Fig. 5. Effects of ISL on VRI-treated ISVs insufficiency in a zebrafish model. (I) At 24 hpf, zebrafish embryos were pre-treated with 575 nM VRI for 4 h, washed and incubated with 0.1% DMSO (negative control; B, B′) or various concentrations of ISL (50–500 μM) for 20 h (C–F, C′–F′). Embryos treated with 0.1% DMSO as control group (A, A′). White arrow indicates mature ISV, red arrow indicates defective ISV. The magnified views of A–F were shown in A′–F′ (higher magnification), respectively. (II) Quantitative analysis showing the concentration-dependent effects of ISL on the recovery of ISVs. Each point represents mean ± SEM ($n = 20$) from a representative experiment. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$. The data were analyzed by one-way ANOVA followed by the Dunnett's test for comparisons of all treated groups with the negative control group. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

cancer therapies and ischemic disorders such as chronic wound [27].

In our *in vivo* zebrafish experiment, we showed that VRI-pretreatment induced blood vessel loss in developing zebrafish accompanied by inactivation of the VEGF signaling pathway. This could be used to identify the pro-angiogenic activity of agent. The concentration of ISL at 500 μM rescued ISV loss on VRI-treated zebrafish embryos, demonstrating superior pro-angiogenic activity without obvious toxicity. This may due to that liquorice can alleviate the toxicity, modulate the taste of herbs and rely its sweet flavor in prescriptions of the Traditional Chinese Medicine (TCM) [12].

Table 1
The effects of inhibitors of VEGF signaling pathways on ISL-induced angiogenesis in VRI-treated zebrafish.

Inhibitors	The rate of inhibition effect		
Apatinib	0.5 μM***	1 μM***	5 μM*** Toxic[a]
LY294002	5 μM***	10 μM***	20 μM*** Toxic[a]
Sorafenib	0.25 μM***	0.5 μM***	1 μM*** Toxic[a]
PD0325901	0.25 μM***	0.5 μM***	1 μM*** Toxic[a]
PP2	2.5 μM ns	5 μM ns	10 μM ns Toxic[a]
P38i	20 μM ns	60 μM ns	100 μM ns Toxic[a]
GDC-0994	20 μM ns	80 μM ns	160 μM ns
MK2206	10 μM ns	20 μM ns	50 μM ns

The semi-quantitative analysis of effects of inhibitors on angiogenesis activity of ISL in VRI-treated zebrafish: ns, no significance; ***, $p < 0.001$ as compared to the ISL-treated zebrafish embryos. The data were analyzed by one-way ANOVA followed by the Tukey's test for comparisons of all treated groups.

[a] Zebrafish embryos exhibit toxicity under the indicated concentration of inhibitor.

Besides, the new vascular vessel brings from ISL exhibited a curved structural morphology, which contribute to the seemingly intact ISVs of ISL-treated group and the a little statistical difference in data compared to the control group after quantification. This kind of vascular structure is similar to tortuous and disorganized vessel networks regenerated after a few months in wound healing model on zebrafish [28]. Angiogenesis undergoes degradation of the vascular basement membrane, migration and proliferation of vascular endothelial cells, lastly characterized by new blood vessel buds branching out from pre-existing blood vessels. This further indicates that the curved and enlarged regenerative blood vessels induced by ISL were fully compliance with the angiogenesis process.

Liquorice is the most widely used herbal medicine. Previous studies have reported that liquorice, especially total flavonoids (liquiritin apioside, liquiritin, isoliquiritin apioside, liquiritigenin and isoliquiritigenin without glycyrrhizic acid) all exist anti-inflammatory activity [29]. ISL have also been reported to possess antioxidant, antigenotoxic activity and anti-depression activities. Notably, a previous study by Shinjiro KOBAYASHI had reported that ISL inhibited angiogenesis in air pouch granuloma angiogenesis on mice model *in vivo* and in primary cultured EC from the thoracic aorta of rats *in vitro*, respectively [30]. The reasons of this discrepancy between the reported article (inhibition) and our results (promotion) could be explained in two aspects. *In vitro*, EC from the thoracic aorta of rats is a kind of aortic, differing from the HUVECs. Vena cava explants exhibited a greater capacity to form neovessels than aortic rings when tested in parallel cultures from the same animal [31]. Besides, the capacity of the aortic is affected by the age and genetic background of the animal significantly, limiting the aortic rings to sprout spontaneously or in response to angiogenic factors [32]. Thus, the EC from the thoracic aorta of rats and HUVECs may exhibit different responses to ISL. *In vivo*, air pouch granuloma model was used for induction of inflammation and derived vascular [33]. This model can quantify pharmacological modulation of angiogenesis in inflammation, which differs from the visualization of vascular development in zebrafish model. In our study, the pro-angiogenic activity of ISL could be observed *in vivo* singularly. In addition, ISL was listed as a component of Gualou Guizhi granules (GLGZG), which could rapidly passed into the bloodstream in middle cerebral artery occlusion (MCAO) rats for the treatment of cerebral ischemia injury [34]. Although it is unclear whether the ISL in Gualou Guizhi granules (GLGZG) exert the pro-angiogenic activity, the potential clinical applications of this compound warrant further investigation.

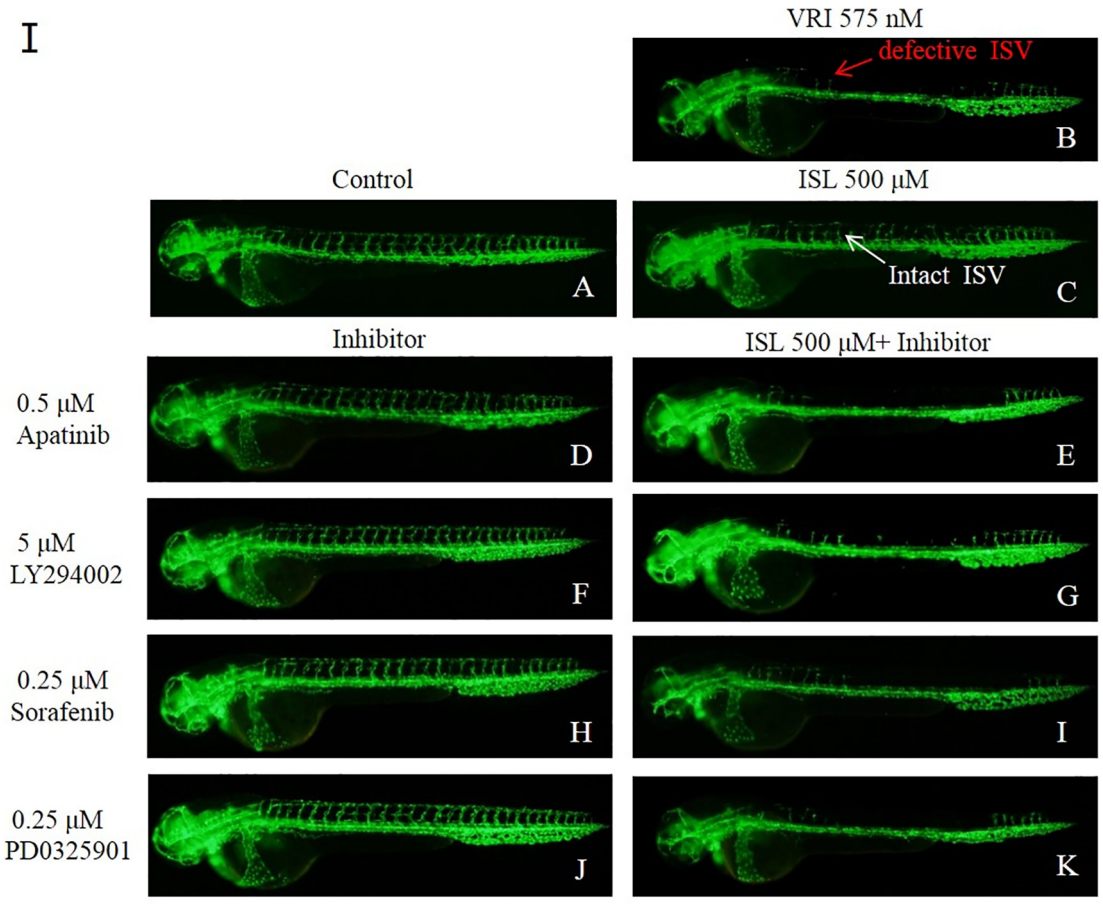
After conforming the pro-angiogenic activity of ISL, further study on the underling mechanism was needed. Angiogenesis requires the involvement of multiple pro-angiogenic factors and is regulated by multiple proteins including cell adhesion molecules, extracellular matric components and vascular endothelial growth factor receptors (VEGFRs) [35,36]. In humans, the VEGF family is composed of several members: VEGF-A, VEGF-B, VEGF-C, VEGF-D, VEGF-E, VEGF-F, placenta growth factor (PLGF) and endocrine gland-derived vascular endothelial growth factor (EG-VEGF) [37]. VEGF-A, which interacts with endothelial cells through VEGFR2, is the main component responsible for the viability and proliferation of endothelial cells [38]. Studies have shown that VEGF-B does not induce neovessel growth or blood vessel permeability under most conditions [39,40]. Additionally, VEGF-B has been shown to act as a critical survival factor that protects cells from death under conditions of tissue/vessel injury [41]. VEGF-C and -D, known as lymphangiogenic growth factors, play an important role in tumor lymphangiogenesis *via* activation of the VEGF receptor (VEGFR)-3, which is expressed in lymphatic endothelial cells [42]. VEGF-C is necessary for lymphatic system, especially for perinatal lymph angiogenesis. The VEGF-C/VEGFR-3 pathway provides a target for treatment of cancer and lymphedema [43].

In our zebrafish blocking assay, we chose eight specific signaling inhibitors of main angiogenesis associated pathways such as p38 MAPK, PI3K, Akt, Src, Raf, MEK, and ERK1/2 which are the downstream signals of VEGFR2 and determined the effects of them against the pro-angiogenic activity of ISL. We found that the pro-angiogenic effects of ISL could be blocked by co-treatment with Raf/MEK, PI3K or VEGFR2 inhibitors but not p38 MAPK, Src, Akt or ERK1/2 inhibitors. These evidences showed that the angiogenesis of ISL probably involved the interaction of VEGFR2, PI3K, Raf/MEK in VRI-treated zebrafish (Fig. 7). *In vivo* model is complicated by cross-talks, synergism and feedback mechanisms between the networks of signaling pathways in angiogenesis for endothelial cell survival, proliferation, differentiation and migration. As known, VEGFRs plays crucial roles in transducing regulatory signal from VEGF which include VEGFR1, VEGFR2 and VEGFR3. Moreover, VEGF and VEGFRs are uniquely required to balance the formation of new blood vessels with the maintenance and remodeling of existing ones during development and in adult tissues [44]. After receptor dimerization and autophosphorylation, several SH2 domain-containing signal transduction molecules are activated either directly such as PLC-gamma, VEGF receptor-associated protein (VRAP), and Sck or by indirect mechanisms, such as Src and PI3K. In our blocking assay, PI3K contributed the promotion of angiogenesis by ISL in VRI-treated blood vessel loss zebrafish. Cell survival signal is mainly mediated through PI3K-mediated activation of Akt/Protein Kinase-B (PKB). Activation of PI3K results in accumulation of phosphatidylinositol-3, 4, 5-Trisphosphate (PIP3), which in turn mediates membrane targeting and phosphorylation of Akt/PKB by binding to its pleckstrin homology (PH) domain [45]. In addition, the Raf/MEK/ERK signaling cascade was used by growth factors and mitogens to transmit signals from their receptors to regulate gene expression and prevent apoptosis [46]. The Raf/MEK/ERK pathway induces events both associated with cell proliferation and cell cycle arrest [47]. In our blocking assay, our results show that the Raf/MEK signaling cascade could reverse all the angiogenic responses induced by ISL. Thus, the PI3K or Raf/MEK may be depended on other signal transducers involved the angiogenesis process.

In general, all the evidences showed the pro-angiogenic activity of ISL probably involved the cross-talk of VEGFR2, PI3K, Raf/MEK signaling pathway in VRI-treated zebrafish. Interference with VEGF function has therefore become of major interest for drug development to regulate angiogenesis and targeting the VEGF signaling pathway may be of therapeutic importance for many diseases.

5. Conclusion

Taken together, these data for the first time showed that ISL, a water soluble component of licorice root, could promoted cell proliferation, migration and tube formation in HUVECs, and rescued ISVs loss on VRI-treated zebrafish *Tg* (*fli-1*: EGFP) embryos. The pro-angiogenic effect of



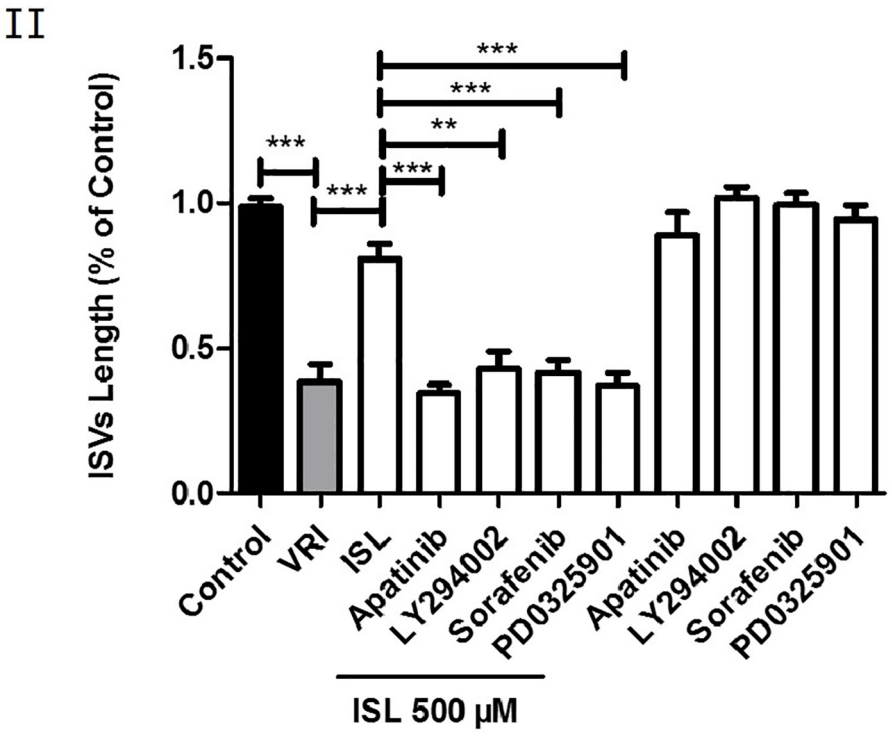


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Fig. 6. The blocking effects of VEGFR2, PI3K, Raf, MEK inhibitors co-treatment with ISL based on ISL-induced angiogenesis in VRI-treated zebrafish. (I) At 24 hpf, *Tg* (*fli:EGFP*) zebrafish embryos were pre-treated with 575 nM VRI for 4 h, washed and incubated with 0.1% DMSO (B) or 500 μM ISL(C), 500 μM ISL and 0.5 μM Apatinib (E), 500 μM ISL and 5 μM LY294002 (G), 500 μM ISL and 0.25 μM Sorafenib (I), 500 μM ISL and 0.25 μM PD0325901 (K) for 20 h. Meanwhile, the sham zebrafish embryos were also treated with 0.1% DMSO (A), 0.5 μM Apatinib (D), 5 μM LY294002 (F), 0.25 μM Sorafenib (H), 0.25 μM PD0325901 (J) for 20 h. White and red arrows indicated that ISVs (intact or defective). (II) Quantitative analysis of inhibitory effect of inhibitors on the recovery of ISVs. Each point represents mean ± SEM ($n = 20$) from a representative experiment. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$. The data were analyzed by one-way ANOVA followed by the Tukey's. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

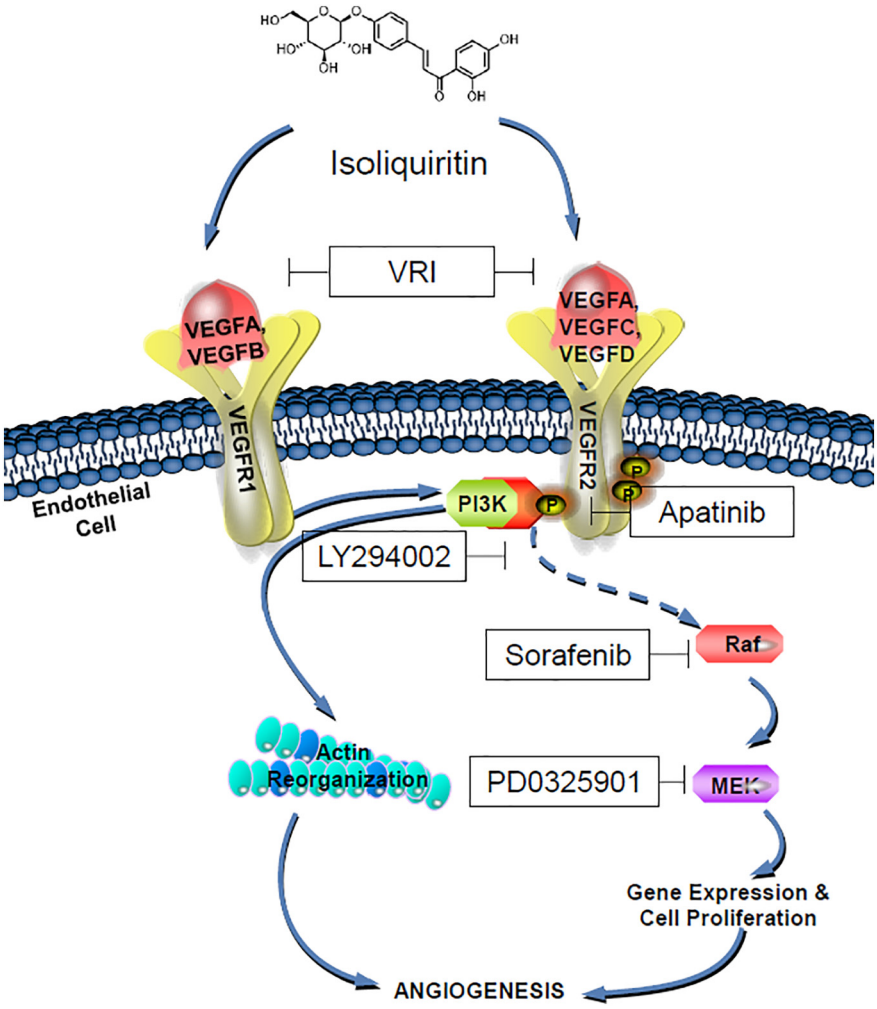

Fig. 7. The proposed interaction of VEGF signaling pathway involved in isoliquiritin-induced angiogenesis effects in VRI-treated zebrafish.

ISL may involve the activation of VEGFR2-, PI3K-, Raf/MEK-dependent signaling pathways. This discovery once again enriched the medicinal value of licorice. It is hoped that our findings inspired a novel research on the pro-angiogenic role during the development of promising drug in cardiovascular diseases, cerebrovascular diseases and other vascular diseases treatment.

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Author contributions statement

Zhang XH and Li CY performed major experiments, Lin QH performed the HUVEC migration and tube formation assay. Feng F and He MF provided the conception and design of the study. Zhang XH, Li CY and He MF participated in the drafting and revision of the manuscript. He ZH helped the language editing and interpretation. He MF obtained funding and supervised the study.

Conflict of interest

Authors declare no conflict of interest.

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