

Phytochemical Isoliquiritigenin Inhibits Angiogenesis *Ex Vivo* and Corneal Neovascularization in Mice

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Abstract

Neovascularization is often involved in many diseases and there is no effective treatment for this pathological process. In searching for potential therapies for neovascularization, we screened nineteen pre-selected small molecules isolated from herbal extracts for their possible anti-angiogenic effect *in vitro* and *in vivo*. We found that isoliquiritigenin, a chalconoid compound isolated from Chinese herb medicine licorice, potently inhibited vascular endothelial cell (EC) proliferation, migration, tube-like structure formation *ex vivo*. Western blot analysis shows that exposure of ECs to isoliquiritigenin inhibited extracellular signal-regulated kinase 1/2 (ERK1/2) phosphorylation. In Matrigel plug assay, isoliquiritigenin effectively blocked fibroblast growth factor-induced *in vivo* angiogenesis in mice. Consistently, topical application of isoliquiritigenin significantly inhibited chemical injury-induced corneal neovascularization in mice. Collectively, these results suggest that isoliquiritigenin may be a low-cost and effective natural agent to treat angiogenesis-dependent diseases.

Keywords: Isoliquiritigenin; Vascular endothelial cell; Proliferation; Migration; Angiogenesis; Neovascularization

Introduction

Angiogenesis is a complex phenomenon long known to be under the tight control by a large panel of cytokines, cells, hormones, etc. [1]. While angiogenesis might occur under both physiological and pathological conditions, neovascularization is often referred to the development of new blood vessels in tissues that are normally not or less vascularized. Except for situations where neovascularization helps to heal and reconstitute the structure of injured tissues, all other instances of neovascularization are associated with the dysfunction of the affected tissues or with the expansion of undesirable tissues (e.g. tumor) [2]. The cornea is normally avascular. Under pathological conditions however, blood vessels from the limbal vascular plexus could invade the cornea, leading to its pathological vascularization that can cause visual impairment and blindness [3,4]. Previous studies showed that corneal neovascularization can be triggered by various factors, including infection [5], trauma [6] and inflammation [7]. While several strategies have been tested for their anti-neovascularization efficacy, such as multiple anti-angiogenic gene therapy [8], recombinant anti-angiogenic factor therapy [9], and anti-inflammation therapy with monoclonal antibody against pro-inflammatory molecules [10], these approaches are not very effective in the treatment of corneal neovascularization [11].

Natural products (e.g. medicinal herbs) have been proposed as alternative resources for anti-angiogenic agents [8,12]. Indeed, traditional Chinese Medicine practitioners have been prescribing herbal medicines to treat various diseases that are associated with neovascularization, although not all of herbal therapies are based on

evidence from the results of modern biomedical studies [13]. In addition, the components that are responsible for the anti-angiogenic action of these herb medicines are largely unknown. In the present study, we first screened 19 small molecules isolated from presumably angiogenesis-modulating herbs for their potential to modulate endothelial cell (EC) behavior *ex vitro*. We then studied the anti-angiogenic effect of the identified compound *in vivo* and further determined the mechanism underlying this action. We found that isoliquiritigenin, a chalconoid compound isolated from Licorice Roots, is a potent agent for the treatment of pathological neovascularization.

Materials and Methods

Herbal isolates

All compounds were isolated and provided by the National Pharmaceutical Engineering Center for Chinese Herbal Medicine in Jiangxi Herbfine Hi-tech Co., Ltd, Nanchang, Jiangxi, China. The purity of all compounds is >98% as determined by high performance liquid chromatography. The compounds were dissolved, either in PBS or dimethyl sulfoxide (DMSO), according to the manufacturer's instructions.

Cell proliferation assay

Primary human umbilical vein endothelial cells (HUVECs) were used for the proliferation analyses. Cell proliferation was measured using the coleregic Cell Counting Kit-8 (CCK, Dojindo laboratories, Kumamoto, Japan) as previously described [14]. In brief, HUVECs were grown to about 30% confluence followed by the addition of the individual compounds or vehicle at a single concentration of 40 μ M. After 48 h of incubation, CCK-8 solution was added and the cells were

incubated for additional 4 h. The absorbance at 450 nm was measured using a microplate reader (Molecular Devices, Sunnyvale, CA, USA).

Cell migration assay

Migration of HUVECs was evaluated by using a monolayer wound healing and the Transwell migration assays. For the wound healing assay, cells were plated on 24-well culture plates and allowed to grow to confluence. A scratch was made with a micropipette tip and the cultures were rinsed to remove detached cells. Cells were then cultured in medium with or without various concentrations of tanshinone IIA or isoliquiritigenin. On the outer surface of the culture wells facing the scratches, ink lines were made perpendicular to the inside scratch and the width of the scratches at the crossing site were photographed before and after the 24 h of incubation. The cell migration rates were calculated as previously described [15].

For Transwell migration assay, HUVECs were placed in the upper compartment of Transwell Boyden chambers with fibronectin-coated polycarbonate filters (8- μ m pore size, Corning Inc., Acton, MA) at 1×10^5 cells/well in 0.2 mL medium containing 1% serum. The lower chamber contained 0.7 mL of complete medium supplemented with increasing concentrations of isoliquiritigenin (0, 25, 50, or 100 μ M). After 6 h of incubation at 37°C, cells remaining on the upper surface of filter were removed using a cotton swab. The cells that had transmigrated to the lower surface of the membranes were stained with 0.1% crystal violet and counted under microscope.

In vitro angiogenesis assay

Thirty μ L of Matrigel[™] Matrix (BD Bioscience, San Jose, CA) was dispensed in 96-well plates and allowed to polymerize for 1 h at 37°C. HUVECs were labeled with Vybrant Dil cell-labeling solution (Invitrogen, Carlsbad, CA, USA) and seeded at a density of 1.5×10^4 cells/well in medium containing various concentrations of isoliquiritigenin (0, 6.25, 12.5, 25, 50 or 100 μ M). Cells were then incubated at 37°C. 6 h later, random fields were photographed using an Eclipse TE2000-U microscope (Nikon, Japan) with a 594 nm filter.

Western blot analysis

Protein extracts from treated and untreated HUVECs were resolved on 10% SDS-PAGE gel and then transferred to nitrocellulose membranes. The membranes were probed with antibody against phosphorylated extracellular signal-regulated kinase 1/2 (ERK1/2; Santa Cruz Biotechnology, Santa Cruz, CA) according to the manufacturer's instructions and the immunoreactive proteins were detected by chemiluminescence. The membranes were then stripped and re-probed with ERK 1/2 antibody (Santa Cruz).

In vivo Matrigel plug angiogenesis assay

C57BL/6 mice (6-10 weeks old) were used for in vivo assays of angiogenesis. Mice were purchased from the Laboratory Animal Center of the Academy of Military Medical Sciences (Beijing, China) and all experimental procedures were carried out in accordance with the guidelines of the Association for Research in Vision and Ophthalmology Statement for the Use of Animals in Ophthalmic and Vision Research. To assess the effect of isoliquiritigenin on angiogenesis *in vivo*, 300 μ L of Matrigel (BD Bioscience, San Jose, CA, USA) containing 60 ng of fibroblast growth factor (R&D System, Minneapolis, MN, USA) and 3U of heparin (B.Braun Melsungen AG,

Melsungen, Germany), with or without 100 μ M of isoliquiritigenin, were injected subcutaneously into the abdominal wall of mice (n=4 mice/group). Ten days after implantation, mice were euthanized and the Matrigel plugs were removed and photographed. Hemoglobin contents in the plugs were measured as described by others [16] with modification. Briefly, the implants were snap frozen, lyophilized overnight, and weighted. The dried Matrigel was re-suspended in 0.4 mL of 0.1% Triton X-100 for 1 h, disrupted by vigorous pipetting, and then centrifuged at $14,000 \times g$ for 15 min to remove any particulate debris. Absorbance of hemoglobin in the supernatants was read at 405 nm with a microplate reader, and its concentration was calculated according to the standard curve obtained with pure hemoglobin.

Corneal neovascularization studies

Under general anesthesia with intraperitoneal injection of ketamine and chlorpromazine (50 mg/kg and 5 mg/kg, respectively), *corneal neovascularization in mice* were generated by application of a 2 mm sized filter soaked with 2 μ L of 1 N NaOH to the central cornea for 40 sec followed by immediate rinsing with normal saline for 40 sec, a widely used mouse model for pathological corneal neovascularization. Isoliquiritigenin or resveratrol (100 μ M in saline eye drops) was applied topically (5 μ L) to the burned eyes 4 times daily for 7 consecutive days. Control eyes were similarly burnt but treated with saline only. At day 7, eyes were photographed under a slit lamp. All mice were killed and their corneas were collected and subjected to conventional histology with hematoxylin-eosin (H&E) staining and immunohistochemistry as detailed below.

Histology and immunohistochemistry

Eyeballs were either fixed with formalin or snap-frozen in Optimal Cutting Temperature (OCT) compound (Sakura Finetech, Tokyo, Japan). Formalin-fixed tissues were stained with H&E. For immunofluorescence staining, cryosections (6 μ m) were prepared from OCT-embedded eyeballs and were fixed in ice-cold acetone for 10 min. The sections were blocked with 10% normal goat serum for 15 min and then stained with PE-conjugated anti-CD31 monoclonal antibody (1:100, BD Biosciences, San Jose, CA, USA) overnight at 4°C. After counterstaining with DAPI, the stained sections were viewed under an Eclipse TE2000-U microscope (Nikon, Japan). Negative controls were stained in parallel by omitting primary antibody.

Reverse-transcription quantitative real-time PCR analysis

Total RNA from mouse corneas was extracted using a NucleoSpin RNAII kit (Macherey-Nagel, Düren, Germany) and reverse transcribed using a PrimeScript RT Reagent Kit (Takara, BIO INC., Shiga, Japan) following the manufacturer's protocols. Quantitative PCR analysis of vascular endothelial growth factor (VEGF), basic fibroblast growth factor (bFGF), and transforming growth factor- β 1 (TGF- β 1) was performed in an ABI 7500 Detection System (Applied Biosystems, Foster City, CA, USA) using TaqMan Mastermix (Tiangen Biotech, Beijing China). RPL5 gene was used as internal control. Reactions were carried out with an initial 10-min incubation at 95°C followed by 45 cycles at 95°C for 15 sec and then at 60°C for 1 min. The raw data were analyzed using SDS 7500 software (Applied Biosystems) and Ct values for each gene in each sample were determined. The relative amount of each target gene mRNA to RPL5 was calculated as the $2^{-\Delta Ct}$ (where $\Delta Ct = Ct - Ct_{RPL5}$) and expressed as folds of control. The primers used in quantitative real-time RT-PCR were listed below (Table 1):

Gene Symbol (Accession Number)	Primer sequences F: forward; R:reverse	Amplicon (bp)
VEGF (NM_009505)	F- GCTACTGCCGTCGATTGAG R- CACACAGGACGGCTTGAAGA	86 bp
bFGF2 (NM_008006)	F- AGAGCGACCCACACGTCAA R- AAGGTACCGTTGGCACACA	86 bp
TGFβ1 (NM_011577)	F- ACGGAATACAGGGCTTTCCA R- GCTGATCCGTTGATTTCCA	86 bp
RPL5 (NM_016980)	F- GGAAGCACATCATGGGTCAGA R- TACGCATCTTCATCTTCTCCATT	70 bp

Table 1: Sequences of primers used for RT-qPCR assay.

Statistical analysis

Data from *in vitro* experiments were derived from at least three independent experiments with duplicated determination each. Variation within treatments is expressed as the standard error (SE) of the mean (SEM) for *in vitro* assays and SE for *in vivo* studies. All data were subjected to a one-way ANOVA using the SigmaPlot software and treatment differences were subjected to Tukey's multiple comparison tests, where $P < 0.05$ was considered significant.

Results

Effects of herb-derived small molecules on EC proliferation

For this study, 19 pure compounds were carefully selected based on the following reasons. First, the herbs from which these molecules were isolated have been used in complex recipes of traditional Chinese Medicine for treating various angiogenesis-related diseases; and second, these selected chemicals had been thought to be bioactive components responsible for some biological effects of corresponding herb(s). These 19 molecules are arteannuinum succinate, baicalein, berberine hydrochloride, ginkgolide B, ginsenoside Rg3, hydroxycamptothecin, isoliquiritigenin, L-epicatechin, ligustrazine hydrochloride, notoginsenoside R1, procyanidin B2, protocatechuic aldehyde, puerarin, quercetin, resveratrol, salviatic acid A sodium, silybin, tanshinone IIA, and wogonin. Beside we first tested their effects on the proliferation of primary human umbilical vascular ECs (HUVECs). In that regard, cells were cultured in the medium with the presence of vehicle or 40 μM various isolated compounds for 24 h. The results showed that baicalein, tanshinone II A, resveratrol, ginsenoside Rg3, arteannuinum succinate, berberine hydrochloride, isoliquiritigenin, and 10-hydroxy camptothecin inhibited cell proliferation, whereas ligustrazine hydrochloride, pueraria, notoginsenoside R1, epicatechin, and silybin stimulated cell proliferation as compared to the control (Figure 1). The order of potency for the inhibition of EC proliferation at this concentration is tanshinone

IIA > isoliquiritigenin > arteannuinum succinate ≈ berberine hydrochloride > hydroxycamptothecin > ginsenoside Rg3 > resveratrol ≈ Baicalein, with tanshinone IIA and isoliquiritigenin blocking cell proliferation by 97.0% and 93.7%, respectively. We further tested dose-dependent effects of tanshinone IIA and isoliquiritigenin on EC proliferation. Both tanshinone IIA (Figure 2A) and isoliquiritigenin (Figure 2B)

dose-dependently inhibited EC proliferation. Tanshinone IIA as low as 5 μM significantly inhibited EC proliferation with 10 μM and 20 μM concentrations blocking cell proliferation by 64% and 94%, respectively, whereas isoliquiritigenin at ≤10 μM concentrations was ineffective with 10 μM reducing proliferation by 42%, suggesting that low-dose tanshinone IIA is more potent than isoliquiritigenin in inhibiting EC proliferation. To confirm that the effect of Tanshinone IIA and isoliquiritigenin in our experiments was on cell proliferation, rather than due to induction of cell death, we assessed if tanshinone IIA and isoliquiritigenin induce apoptosis of HUVECs. In that regard, cells were treated with various doses of tanshinone IIA or isoliquiritigenin for 24 h. The results showed that tanshinone IIA or isoliquiritigenin ISL at ≤80 μM had no effect on cell apoptosis (data not shown). Since the long-term purpose of this study is to identify herb-derived small molecules that will be potentially used in managing human neovascularization diseases, we focused following studies on these two most potent compounds that inhibited HUVEC proliferation.

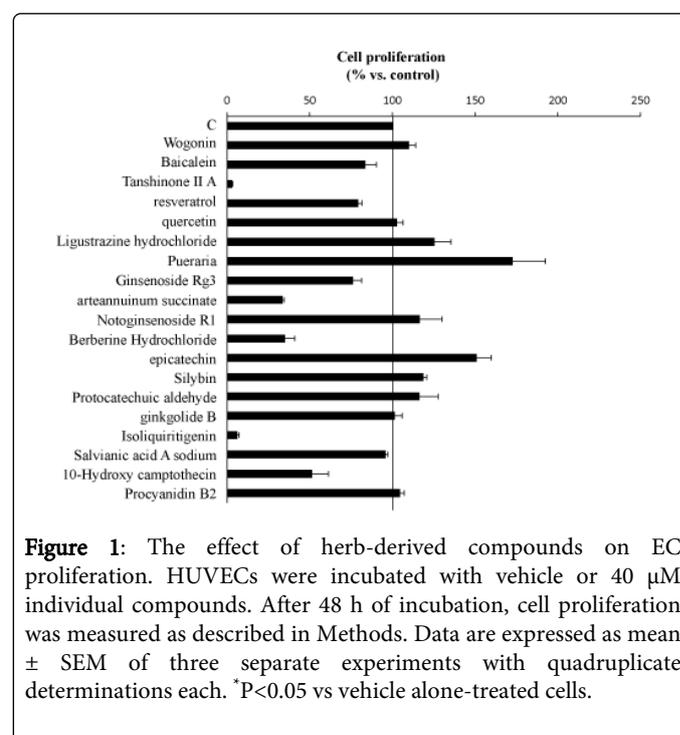


Figure 1: The effect of herb-derived compounds on EC proliferation. HUVECs were incubated with vehicle or 40 μM individual compounds. After 48 h of incubation, cell proliferation was measured as described in Methods. Data are expressed as mean ± SEM of three separate experiments with quadruplicate determinations each. * $P < 0.05$ vs vehicle alone-treated cells.

Effects of tanshinone IIA and isoliquiritigenin on EC migration

The chemotactic migration of ECs is one of the critical steps in the process of angiogenesis. We therefore examined whether tanshinone IIA and isoliquiritigenin can further inhibit migration of HUVECs. We first performed the monolayer wound healing assay to evaluate whether these compounds affect EC migration. The results showed that both tanshinone IIA and isoliquiritigenin dose-dependently inhibited cell migration from the edge of the wound into the open area, but isoliquiritigenin is more potent than Tanshinone IIA in inhibiting the migration of HUVECs (Figure 3A and 3B). Next, we performed transwell assays by using modified Boyden chambers to further evaluate whether isoliquiritigenin affects cell migration in HUVECs. As shown in Figure 3C, isoliquiritigenin significantly

reduced the number of migrated cells through the membrane barrier of the transwell.

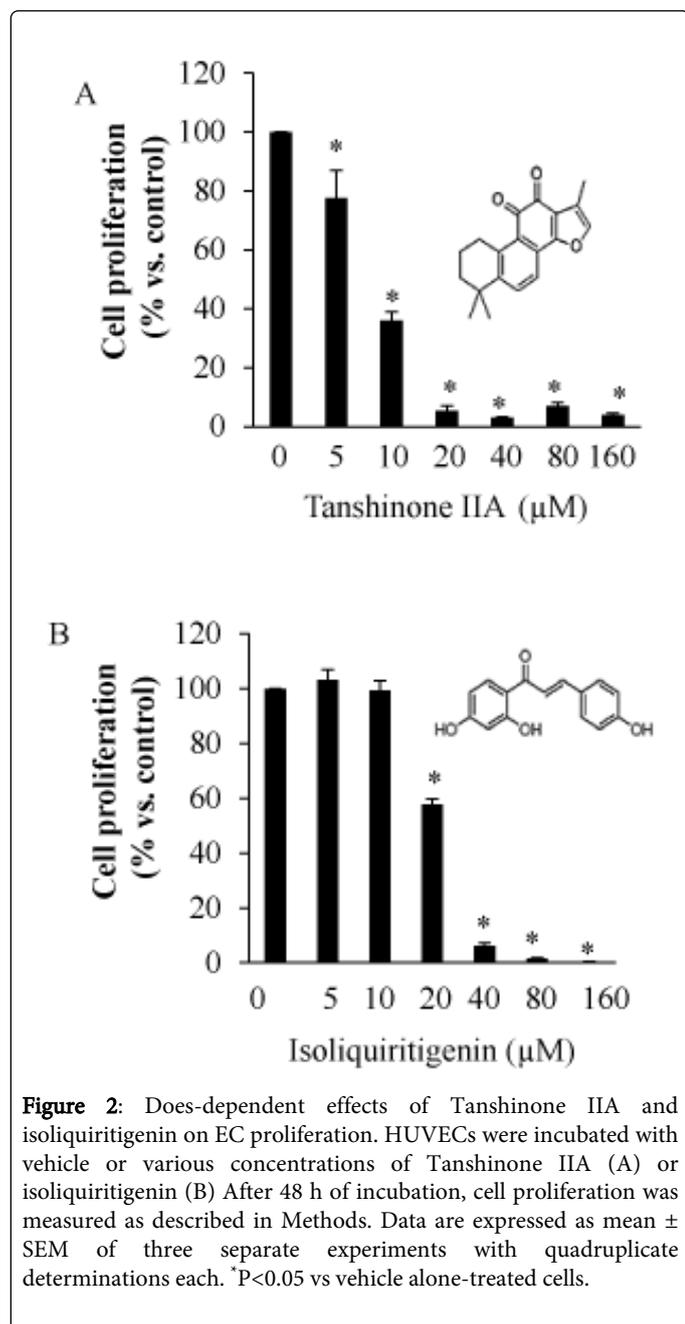


Figure 2: Dose-dependent effects of Tanshinone IIA and isoliquiritigenin on EC proliferation. HUVECs were incubated with vehicle or various concentrations of Tanshinone IIA (A) or isoliquiritigenin (B) After 48 h of incubation, cell proliferation was measured as described in Methods. Data are expressed as mean ± SEM of three separate experiments with quadruplicate determinations each. *P<0.05 vs vehicle alone-treated cells.

Isoliquiritigenin abolishes in vitro angiogenesis

Angiogenesis is a complex process involving EC proliferation, migration, and the formation of vascular tube structures. Next, we evaluated if isoliquiritigenin affects EC tube structure formation, a widely used in vitro model of angiogenesis. In that regard, cells were plated onto Matrigel, followed by incubation with or without isoliquiritigenin. As shown in Figure 4, ECs developed tube/cord-like networks after 6 h of incubation. However, exposure of cells to isoliquiritigenin substantially ablated tube-like structure formation.

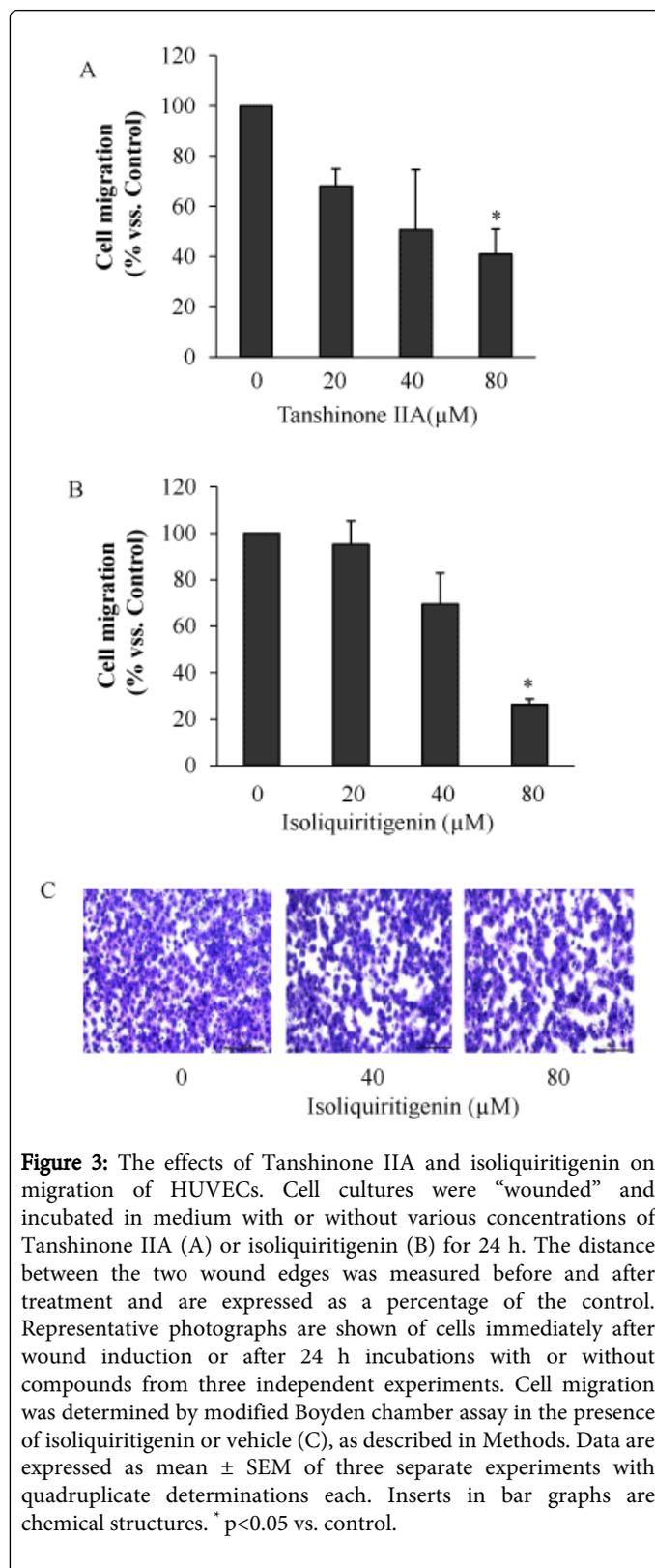


Figure 3: The effects of Tanshinone IIA and isoliquiritigenin on migration of HUVECs. Cell cultures were “wounded” and incubated in medium with or without various concentrations of Tanshinone IIA (A) or isoliquiritigenin (B) for 24 h. The distance between the two wound edges was measured before and after treatment and are expressed as a percentage of the control. Representative photographs are shown of cells immediately after wound induction or after 24 h incubations with or without compounds from three independent experiments. Cell migration was determined by modified Boyden chamber assay in the presence of isoliquiritigenin or vehicle (C), as described in Methods. Data are expressed as mean ± SEM of three separate experiments with quadruplicate determinations each. Inserts in bar graphs are chemical structures. *p<0.05 vs. control.

Isoliquiritigenin may inhibit angiogenesis through suppression of ERK1/2 activity

It is well recognized that the ERK 1/2 signaling pathway plays an important role in cell proliferation and growth. Next, we investigated the possible pathways involved in the modulation of angiogenesis by comparing the total and activated ERK1/2 levels in isoliquiritigenin-treated and untreated HUVECs. The results from Western blot analysis showed that isoliquiritigenin induced a dose-dependent inhibition of the phosphorylation of ERK1/2, a magnitude similar to its effect on cell proliferation and migration (Figure 5). However, isoliquiritigenin had no effect on total ERK protein expression in the cells.

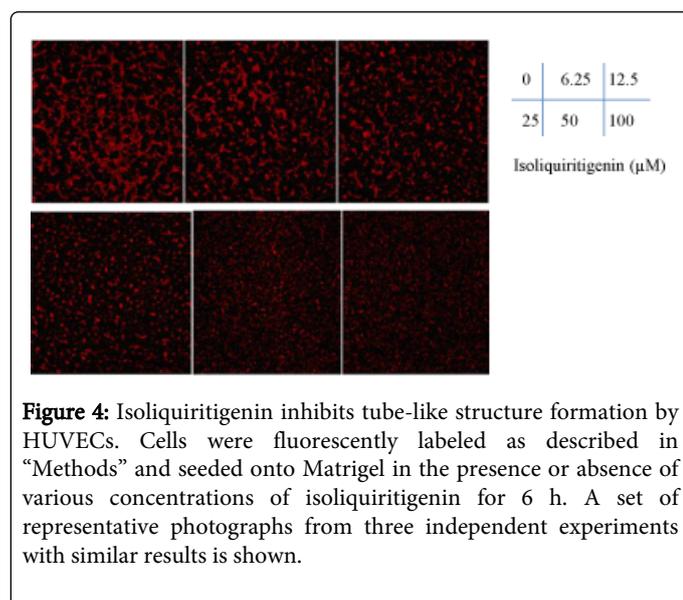


Figure 4: Isoliquiritigenin inhibits tube-like structure formation by HUVECs. Cells were fluorescently labeled as described in “Methods” and seeded onto Matrigel in the presence or absence of various concentrations of isoliquiritigenin for 6 h. A set of representative photographs from three independent experiments with similar results is shown.

Isoliquiritigenin suppresses vasculogenesis and corneal neovascularization

To confirm the anti-angiogenic activity of isoliquiritigenin *in vivo*, we first used the mouse Matrigel plug model of angiogenesis. Resveratrol was chosen as the positive control because it is often used as an anti-angiogenic agent to treat various angiogenesis-related diseases [17-19]. As shown in Figure 6A, significant blood vessel formation was observed in Matrigel plugs supplemented with fibroblast growth factor (FGF) as compared to the plugs harvested from the control mice. However, microvessel formation induced by FGF was profoundly reduced in the presence of 100 μM isoliquiritigenin, as demonstrated by relatively pale color of isoliquiritigenin-supplemented plugs compared to that of vehicle-treated plugs, which was more effective than that of resveratrol treatment (Figure 6A). Consistently, isoliquiritigenin potently reduced the hemoglobin content of the plugs (Figure 6B), further confirming that isoliquiritigenin is a potent inhibitor of growth factor-induced angiogenesis *in vivo*.

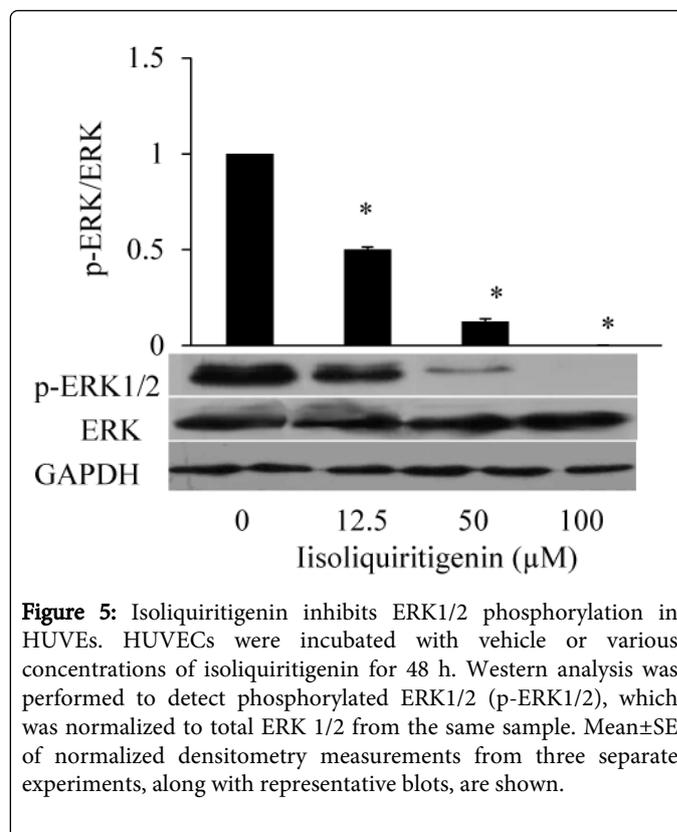


Figure 5: Isoliquiritigenin inhibits ERK1/2 phosphorylation in HUVECs. HUVECs were incubated with vehicle or various concentrations of isoliquiritigenin for 48 h. Western analysis was performed to detect phosphorylated ERK1/2 (p-ERK1/2), which was normalized to total ERK 1/2 from the same sample. Mean±SE of normalized densitometry measurements from three separate experiments, along with representative blots, are shown.

To further test the therapeutic potential of isoliquiritigenin in treating corneal neovascularization, we generated a mouse model of corneal neovascularization by alkali burning of the mouse cornea, which causes injury to the corneal surface and subsequent neovascularization, a well-established model for the study of corneal neovascularization. As shown in Figure 7A, topical application of isoliquiritigenin (100 μM in eye drops) to the corneas injured by chemical burn greatly inhibited the development of corneal neovascularization, an effect that was more potent than that of resveratrol. Analysis of sections from H&E (Figure 7B) and immunohistochemistry staining (Figure 7C) further showed that isoliquiritigenin greatly decreased the numbers of blood/lymphatic vessels in the corneas, which was also more effective than that of resveratrol.

Isoliquiritigenin has no effect on the gene expression of angiogenic factors in cornea

It is recognized that overexpression of several growth factors in the cornea, including vascular endothelial growth factor (VEGF), basic fibroblast growth factor (bFGF) and transforming growth factor beta1 (TGFβ1), plays pivotal roles in promoting angiogenesis and corneal neovascularization [3,20-22], we then performed RT-PCR to determine whether isoliquiritigenin suppresses neovascularization through suppressing the production of angiogenic factors. The results show that chemical burn drastically increased gene expression of VEGF, bFGF, and TGFβ1 by 11.8, 142.9 and 3.2 fold, respectively, in the neovascularized corneas from mice. However, neither isoliquiritigenin nor resveratrol significantly affected chemical injury-induced up regulation of these angiogenic factors (data not shown).

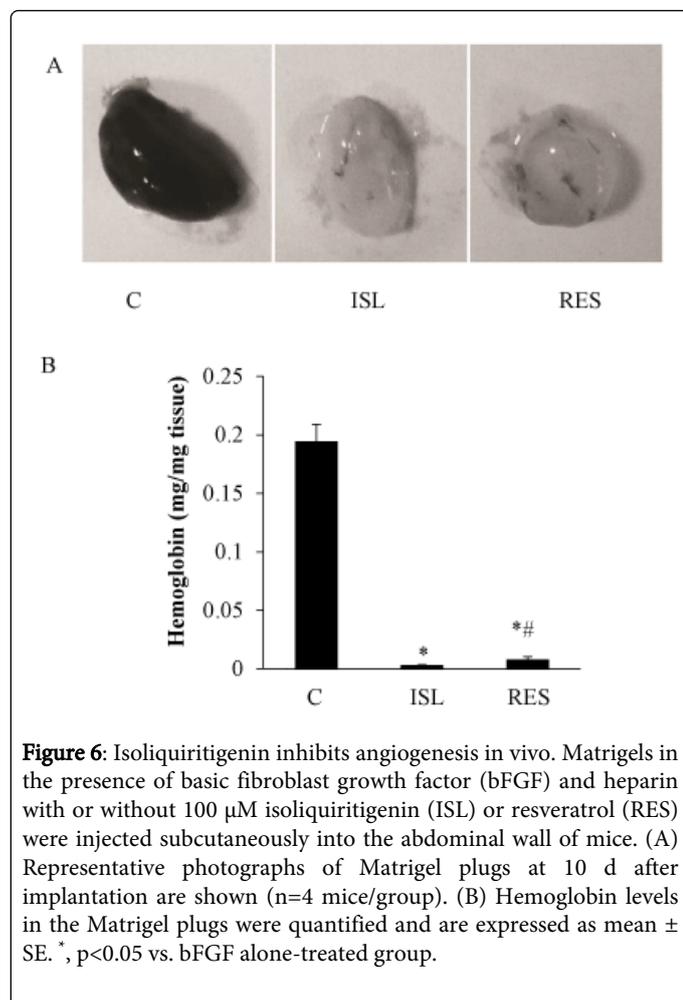


Figure 6: Isoliquiritigenin inhibits angiogenesis in vivo. Matrigels in the presence of basic fibroblast growth factor (bFGF) and heparin with or without 100 μ M isoliquiritigenin (ISL) or resveratrol (RES) were injected subcutaneously into the abdominal wall of mice. (A) Representative photographs of Matrigel plugs at 10 d after implantation are shown (n=4 mice/group). (B) Hemoglobin levels in the Matrigel plugs were quantified and are expressed as mean \pm SE. *, p<0.05 vs. bFGF alone-treated group.

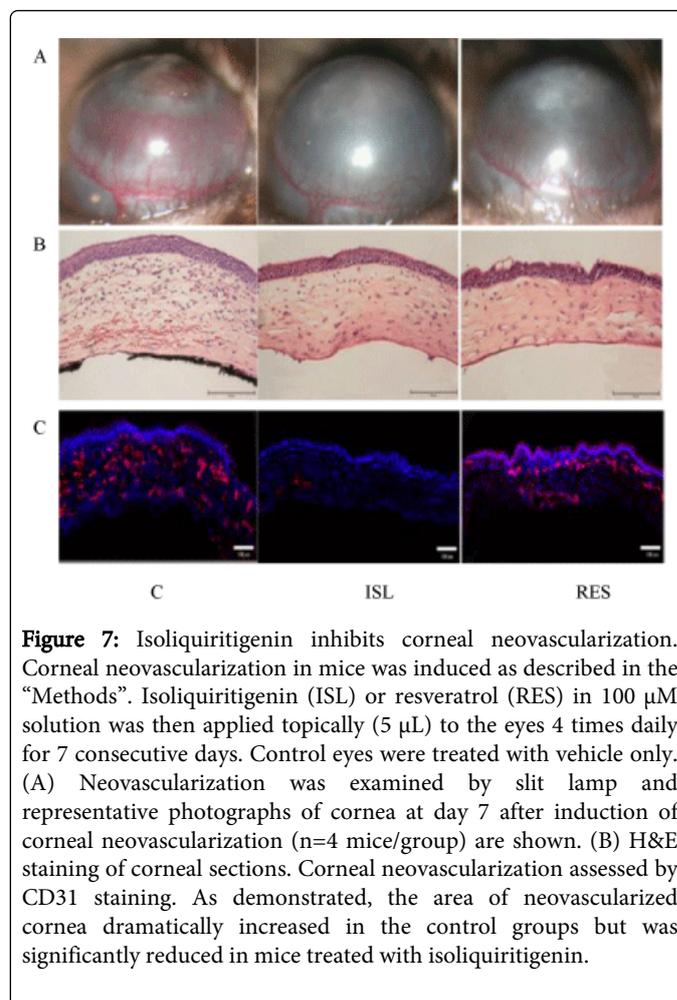


Figure 7: Isoliquiritigenin inhibits corneal neovascularization. Corneal neovascularization in mice was induced as described in the “Methods”. Isoliquiritigenin (ISL) or resveratrol (RES) in 100 μ M solution was then applied topically (5 μ L) to the eyes 4 times daily for 7 consecutive days. Control eyes were treated with vehicle only. (A) Neovascularization was examined by slit lamp and representative photographs of cornea at day 7 after induction of corneal neovascularization (n=4 mice/group) are shown. (B) H&E staining of corneal sections. Corneal neovascularization assessed by CD31 staining. As demonstrated, the area of neovascularized cornea dramatically increased in the control groups but was significantly reduced in mice treated with isoliquiritigenin.

Discussion

Angiogenesis is a complex process with EC cell proliferation, migration, and tubulogenesis playing an essential role in ultimate new blood vessel formation [15]. Corneal angiogenesis causes vascularization of avascular cornea that can lead to the decline of vision and even blindness. The pathogenesis of corneal neovascularization is still not fully understood, but it is stimulated by various growth factors, notably VEGF and bFGF that are secreted in response to hypoxia, chemical burn, chronic inflammation, infection, and trauma [22]. Isoliquiritigenin is a naturally occurring flavonoid that reportedly has an array of biological activities, such as vasorelaxation [23], anti-platelet aggregation [24], anti-inflammation [25], and anti-cancer [26]. While studies examining the effects of this compound on vascular ECs are limited, a recent study showed that isoliquiritigenin suppressed growth factor-stimulated proliferation of ECs [27]. In the present study, we identify that isoliquiritigenin potently inhibits EC proliferation, migration, and the formation of tube-like structure *ex vivo*. Consistently, we demonstrate that this compound is a strong inhibitor of angiogenesis and corneal neovascularization induced by growth factor and chemical injury in vivo. These data suggest that isoliquiritigenin may have great therapeutic for ocular neovascularization and other disorders associated with angiogenesis.

The exact mechanism for isoliquiritigenin to inhibit corneal neovascularization is presently unclear. It is well recognized that the ERK1/2 signaling pathway in vascular ECs plays an important role in cell proliferation, migration, and angiogenesis [28,29]. ERK1/2 are activated in response to a large array of extracellular stimuli, including growth factors [29-31]. In the present study, we show that isoliquiritigenin greatly suppressed ERK1/2 activation, suggesting that its inhibitory action on angiogenesis may be primarily mediated via inhibiting the ERK1/2 signaling pathway. Interestingly, isoliquiritigenin treatment had no effect on ERK1/2 protein expression, suggesting that isoliquiritigenin ablates ERK1/2 activity via a non-genomic mechanism. ERK1/2 is directly activated by MEK, which is activated by its upstream kinase, Raf-1 [32]. However, how isoliquiritigenin interacts with the ERK 1/2 pathway remains to be elucidated.

Accumulating evidence show that several growth factors, particularly VEGF and bFGF, play a critical role in corneal angiogenesis [3,20,21]. Indeed, studies showed that these angiogenic factors are over-expressed and increasingly secreted from neovascularized cornea [6]. Accordingly, corneal neovascularization can be reduced by anti-VEGF or anti-bFGF treatment [22,33]. In the present study, our data show that the expression of these angiogenic factors in neovascularized mouse cornea was greatly increased. However, treatment with isoliquiritigenin had no significant effect on these molecules, suggesting that the anti-angiogenesis action of

isoliquiritigenin is not mediated through suppressing the expression of VEGF and bFGF in the cornea.

Tanshinone IIA, isolated from the root of *Salvia miltiorrhiza*, is widely used in the prescriptions for treating vascular diseases. While the scientific evidence supporting its beneficial effect on vasculature in humans is not well established, some studies showed that tanshinone IIA protects against vascular inflammation and promotes vasodilatation [34-36]. In addition, tanshinone IIA has been shown to inhibit proliferation and migration of cancer cells [37-39]. In the present study, we demonstrate that tanshinone IIA is a potent agent inhibiting EC proliferation and does-dependently inhibits cell migration, but it is less potent than isoliquiritigenin in inhibiting EC migration, which is essential to angiogenesis. Given this result, we then solely focused on further characterizing the anti-angiogenic action of isoliquiritigenin. However, there is possibility that tanshinone IIA is still a potent anti-angiogenesis agent *in vivo*, as it may be able to suppress angiogenesis by inhibiting migration of smooth muscle cells, as reported [40].

In summary, by screening herb-derived small molecules, we identified that isoliquiritigenin is a potent inhibitor of angiogenesis and corneal neovascularization *in vivo*. These effects are ascribed at least in part to the inhibition of proliferation, migration, and tubulogenesis of ECs. These data suggest that isoliquiritigenin may have great therapeutic potential as a low-cost natural compound for neovascularization-associated corneal disorders or other diseases. However, the detailed anti-angiogenic mechanisms of isoliquiritigenin remain to be determined.

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