약학박사 학위논문

Mechanism for Anti-angiogenic Activity of Wondonin Analogues

Wondonin 유도체의 혈관신생 억제 작용기전 연구

2017년 8월

서울대학교 대학원

약학과 천연물과학전공

오 제 도

Mechanism for Anti-angiogenic Activity of Wondonin Analogues

Wondonin 유도체의 혈관신생 억제 작용기전 연구

지도 교수 이 상 국

이 논문을 약학박사 학위논문으로 제출함
2016 년 12 월

서울대학교 대학원
약학과 천연물과학전공
오 제 도

오제도의 약학박사 학위논문을 인준함
2016 년 12 월

위 원 장 박 성 혁 (인)

부위원장 노 민 수 (인)

위 원 김 상 희 (인)

위 원 진 영 원 (인)

위 원 이 상 국 (인)

Abstract

Mechanism for Anti-angiogenic Activity of Wondonin Analogues

Oh Jedo

Natural Products Science Major

College of Pharmacy

The Graduate School

Seoul National University

Angiogenesis is the process which is essential in development of the adult or the fetus. But pathologic angiogenesis (excessive or insufficient) is becoming the cause of various disease which like cancer, age-related macular degeneration (AMD), diabetic retinopathy and cardio vascular disease etc. Thus, many research progress about treatment of diseases which is caused by with unusual blood vessel formation.

Next, synthesized derivatives which base on the wondonin from marine organism. So, excellent anti-angiogenic effect was investigated by these wondonin analogues.

In this research tried to anti-angiogenic effect and mechanism of wondonin analogues. Therefore, this study searching for the derivative which it doesn't have toxicity in cell and effectively suppresses angiogenesis.

First, activities and SI values of analogues were compared with those of wondonin.

And then, analogues were sorted through tube formation assay which is index of new blood vessel formation and cytotoxicity assay.

Among the wondonin analogues, ST-XII-145 was effectively suppressed PI3K/AKT/eNOS, FAK and p38 protein expression in VEGF-induced condition and hypoxic condition. The based on like this in-vitro result and then performed mouse aorta ring assay (*ex-vivo*) and zebrafish angiogenesis assay (*in-vivo*). In this result, it had able to observe an angiogenesis suppression ability.

To investigation of suppression of blood vessel formation in generating process, used mouse embryonic stem cell as differentiate endothelial cells. And then, it used immunocytochemistry methods which anti-angiogenic activity of differentiated endothelial cell and it confirms. After, attempted finding to new analogues that improved activity to stand on the basis of ST-XII-145.

In order to lower the cytotoxicity and raise activity, led to analogues which are various it synthesized by structural change. Selected ST2-VI-66 among the synthesized analogues because SI value and activity are highest. When investigated the molecular targets of anti-angiogenic activity of ST2-VI-66, it suppressed PI3K-AKT-eNOS and FAK signaling pathway.

Furthermore, in order to secure another applicability of diseases treatment which leads angiogenesis suppression the diabetic retinopathy disease model in zebrafish it applied. As a result, ST2-VI-66 effectively suppressed vessel of expanded by high-glucose.

Herein, wondonin derivatives suppressed VEGF signal transduction systems and it presents the possibility as the candidate compound it will be able to overcome the

diseases which occur with the angiogenesis.

Keywords: Angiogenesis, Wondonin analogues, VEGF signaling, Human umbilical vein endothelial cells (HUVEC), mouse embryonic stem cell (mES)/embryonic body (EB), Zebrafish

Student Number: 2012-21599

Table of Contents

List of Tables

List of Figures

I. General Introduction

A. Background

1. Angiogenesis

Angiogenesis, essential phase in embryonic and adult development, generates the formation of new blood vessel from pre-existing vessel. Angiogenesis is composed of the process which sequential complicated the various phase. First, sprouting angiogenesis induce enzymatic degradation of capillary basement membrane and then secretion of various angiogenic growth factor. Next process is endothelial cell (EC) proliferation, directed migration of ECs, tubulogenesis (EC tube formation), vessel fusion, vessel pruning and finally pericyte stabilization (Adair and Montani, 2010).

But, in the pathological conditions, the angiogenesis process gets out of control which results in the development and growth of primary tumors and also increases the risk of metastasis (King et al., 2014; Kinja et al., 2011; Shahneh et al., 2013).

This phenomenon can be found progression of cancer and ophthalmologic disease (Folkman and Klagsbrun, 1987). Pathologic angiogenesis (excessive or insufficient) is leading to various disease. Such as, excessive angiogenesis induces common denominator underlying a number of deadly and debilitating human diseases, including cancer, age-related macular degeneration (AMD), diabetic retinopathy, psoriasis, rheumatoid arthritis and chronic inflammation. On the other hand, insufficient angiogenesis induces coronary artery disease, stroke, ulcer, delayed

wound healing and myocardiac infarction (Carmeliet and Jain, 2000, 2011; Chung et al., 2010).

Especially, among excessive induced disease, one of the most involved factors in cancer invasion and metastasis is angiogenesis among excessive induces disease (Lenzi et al., 2016).

Another disease, intraocular neovascularization develops in response to retinal ischemia and includes neovascularization of the retina, neovascularization of the optic disc, or neovascularization of the iris (Miller, 1997). Neovascularization is a main pathologic characteristic of proliferative diabetic retinopathy (DR) and of retinopathy of prematurity, sickle cell disease, and radiation (McIntosh et al., 2010). The microvascular lesions in DR include thickening of the capillary basement membrane, loss of pericytes and vascular smooth muscle cells, microaneurysms, and capillary occlusions and acellularity (Curtis et al., 2009). As the disease progresses, neovascularization develops in response to inner retinal ischemia. Capillary non-perfusion leading to retinal ischemia and neovascularization also may follow radiation damage of the endothelium of the retinal capillaries (Archer, 1993). The current approved drugs of anti-angiogenesis are summarized in Table 1.

Table 1. Approved anti-angiogenic drugs (De Falco S, 2014)

Drug/company	Type of inhibitor	Targets	Indication
Bevacizumab (Avastin) Genentech/Roche	Monoclonal Antibody	VEGF-A	Metastatic colorectal cancer Nonsmall cell lung cancer Recurrent gliobastoma Metastatic renal cell carcinoma Wet age-related macular degeneration Macular edema following CRVO
Vatalanib (Caprelsa) Astra Zeneca	Tyrosine kinase inhibitor	VEGFRs, EGFR, RET	Last-stage medullary thyroid cancer
Aflibercept (Zaltrap) Regeneron/Sanofi	Chimeric soluble receptor	VEGF-A, VEGF-B, PIGF	Metastatic colorectal cancer
Sorafenib (Nexavar) Bayer/Onyx	Tyrosine kinase inhibitor	VEGFR, PDGFRs, FGFR-1, KIT, RAF	Metastatic renal cell carcinoma Unresectable hepatocellular carcinoma
Sunitinib (Sutent) Pfizer	Tyrosine kinase inhibitor	VEGFRs, PDGFRs, KIT, FLT-3	Metastatic renal cell carcinoma Gastrointestinal stromal tumor Unresectable pancreatic neuroendocrine tumor
Pegaptanib (Macugen) Eyetech Pharms	Pegylated aptamer	VEGF-A_{165}	Wet-age related macular degeneration
Ranibizumab (Lucentis) Genetech/Roche	Fab fragment of antibody	VEGF-A	Wet-age related macular degeneration Macular edema following CRVO
Aflibercept (Eleya) Regeneron/Bayer	Chemeric soluble receptor	VEGF-A, VEGF-B, PIGF	Wet-age related macular degeneration Macular edema following CRVO
Pazopanib (Votrient) GlaxoSmithKline	Tyrosine kinase inhibitor	VEGFRs, PDGFRs, KIT	Metastatic renal cell carcinoma Advanced soft tissue sarcoma

Table 1. Continued

Drug/company	Type of inhibitor	Targets	Indication
Axitinib (Inlyta) Pfizer	Tyrosine kinase inhibitor	VEGFRs, PDGFRs, KIT	Metastatic renal cell carcinoma
Cabozantinib (Cometriq) Exelixis	Tyrosine kinase inhibitor	VEGFR-2, RET, MET	Progressive medullary thyroid cancer
Regorafenib (Stivarga) Bayer/Onyx	Tyrosine kinase inhibitor	VEGFRs, TIE-2, PDGFRs, RET, KIT, FGFRs	Metastatic renal cell carcinoma Gastrointestinal stromal tumor

VEGF-A, vascular endothelial growth factor A; CRVO, central retinal vein occlusion; VEGF-B, vascular endothelial growth factor B; PlGF, placental growth factor; VEGFR, vascular endothelial growth factor receptor; PDGFR, platelet derived growth factor receptor; FGFR, fibroblast growth factor receptor; KIT, v-kit feline sarcoma viral oncogene homolog; RAF, v-raf-1 murine leukemia viral oncogene homolog 1; FLT-3, fms-related tyrosine kinase 3; EGFR, epidermal growth factor receptor; RET, ret proto-oncogene; MET, met proto-oncogene; TIE, TEK tyrosine kinase endothelial.

2. VEGF signaling pathway

Vascular endothelial growth factors (VEGFs) are major regulators of vascular development not only during embryogenesis (vasculogenesis) but also blood-vessel formation (angiogenesis) in the adult. VEGF family is composed of five members, VEGFA, B, C, D and placenta growth factor (PLGF). VEGF A, B and PLGF bind to VEGFR1, VEGFA and E bind to VEGFR2, and VEGFC and D bind to VEGFR3.

The important role of VEGFR2 signaling during development and in neovascularization in physiological or pathological conditions *in vivo* has allowed the design of clinically beneficial therapies (Olsson et al., 2006).

Only a few SH2-domain-containing molecules have been shown to interact directly with VEGFR2. PLCγ binds to phosphorylated Tyr1175 (Tyr1173 in the mouse), and mediates the activation of the mitogen-activated protein kinase (MAPK)/extracellular-signal-regulated kinase-1/2 (ERK1/2) cascade and proliferation of endothelial cells (Takahashi et al., 2001). Beside, PLCγ, the adaptor molecule Shb binds to phosphorylated Tyr1175. VEGF-induced activation of PI3K and then AKT/PKB is activated downstream of PI3K that mediates survival of the endothelial cells (Dayanir et al., 2001; Fujio and Walsh, 1999). Also, activation of endothelial NO synthesis (eNOS) is regulated by AKT/PKB. And then, nitric oxide (NO) synthesize is regulated by eNOS (Olsson et al., 2006).

In human umbilical-vein endothelial cells (HUVECs), Ras is activated by VEGF stimulation and has been coupled to an angiogenic phenotype of endothelial cells (Meadows et al., 2001; Shu et al., 2002).

VEGFR2 (Tyr951) is another important phosphorylation site, which is a binding site for the signaling adaptor TSAd (T-cell-specific adaptor). The endothelial cell migration is regulated by phosphorylation of Tyr951-TSAd pathway (Matsumoto et al., 2005; Zeng et al., 2001).

The formation of complex between TSAd and Src induced by VEGFA, which suggests that Src activation regulated by TSAd and vascular permeability downstream of VEGFR2 (Matsumoto et al., 2005).

Activation of p38 MAPK induce actin-remodeling. Moreover, the heat-shock protein-27 (HSP27) phosphorylation is induced by p38 MAPK, these molecular chaperon that positively regulates VEGF-induced actin reorganization and migration (McMullen et al., 2005; Rousseau et al., 1997).

Also, the focal-adhesion kinase (FAK) and its substrate paxillin are other signaling molecules that involved in focal-adhesion turnover during cellular migration (Abedi and Zachary, 1997; Le Boeuf et al., 2004).

Therefore, multiple signaling pathways converge in distinct patterns eventually resulting in different biological responses such as angiogenesis. Combined stimulation of ERK, Akt, Src, FAK and family G protein seems to be required in angiogenesis, whereas Src-kinases, Rac G protein, phosphatases and eNOS are essential for vascular permeability (Claesson-Welsh and Welsh, 2013).

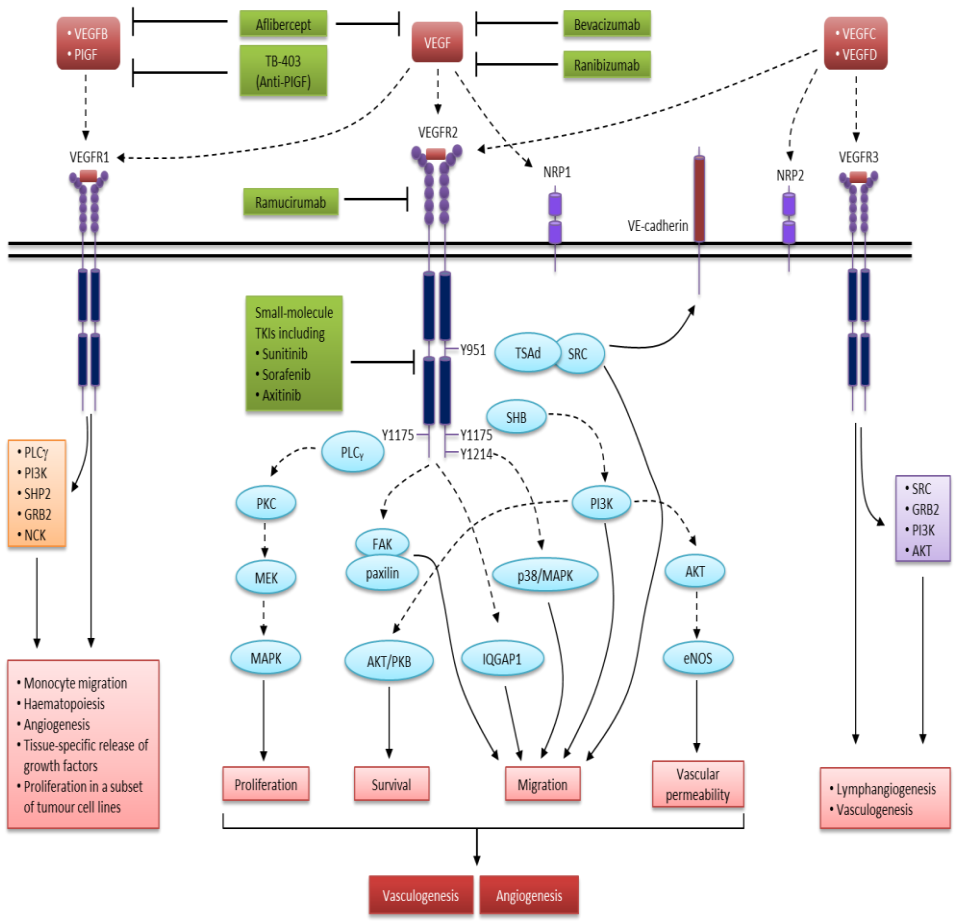


Figure 1. VEGF signaling pathways and inhibitors

3. HIF signaling

Hypoxia-inducible factor (HIF) is a transcription factor that is found in mammalian cells cultured under lower oxygen tension and that plays a key role in the cellular response to hypoxia. HIF is a heterodimer consisting of two subunits, an oxygen-sensitive HIF-α and a constitutively expressed HIF-β (Wang et al., 1995).

In normoxic conditions, HIF-1α is subject degradation by process of pVHL(von HippeleLindau)-mediated ubiquitin-proteasome pathway. On the other hand, hypoxia blocks degradation of HIF-1α leading to accumulation (Huang et al., 1996; Kallio et al., 1997).

The avascular tumor or stroma cells in hypoxic condition pathologically or physiologically triggers angiogenesis as a consequence of an oxygen sensing mechanism and subsequent induction of a variety of pro-angiogenic genes (Bunn and Poyton, 1996; Giordano and Johnson, 2001; Semenza, 2000a).

Vascular endothelial cell growth factor (VEGF) is one of the major target genes, specifically gathers endothelial cells into hypoxic area and stimulates proliferation of these cells. VEGF is known to directly affect to angiogenesis and this growth factor interacts with its receptor, VEGFR, which is specifically expressed in endothelial cells, and stimulates endothelial cell proliferation (Conway et al., 2001; Harris, 2000; Josko et al., 2000; Neufeld et al., 1999).

It was also shown that hypoxia induces the mRNA and protein expression of VEGF, suggesting that up-regulation of the VEGF expression through the hypoxia is stimulation of angiogenesis (Ahmed et al., 2000; Harris, 2000; Neufeld et al.,

1999). Therefore, HIF-1 contributes to angiogenesis through VEGF induction (Wenger, 2002).

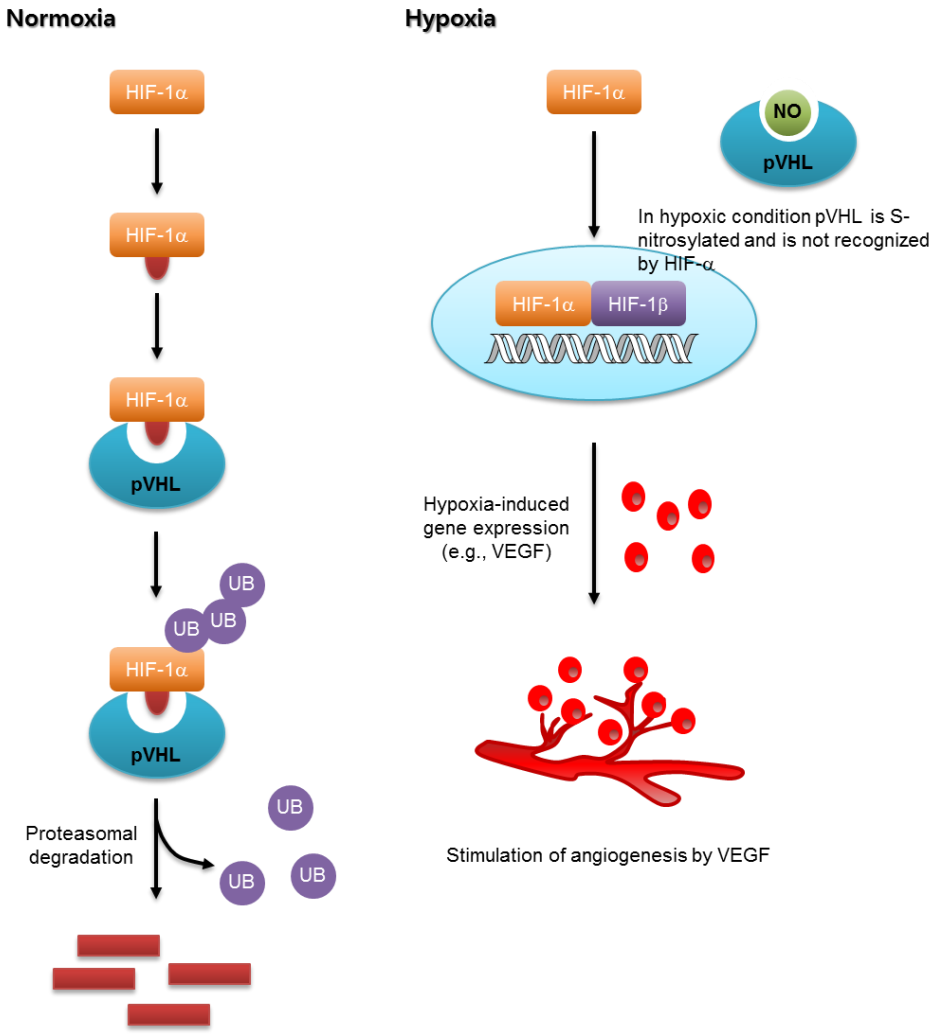


Figure 2. Regulation of HIF signaling

4. Natural products as sources of anti-angiogenesis

During the past decade, intensive studies in plant material have led to the confirmation of antiangiogenic properties and antitumor activity of a number of commonly used medicinal plants (Fresco et al., 2006; Lau et al., 2008; Lee et al., 2008a). Plant-derived substances are characterized by expected properties in terms of antitumor activity, gaining considerable interest among many synthetic chemotherapeutic agents used in oncology (Cragg and Newman, 2013; de Vogel et al., 2008; Mehta et al., 2014). Both untreated plant extracts and purified individual substances are used, which through a number of specific arteries in tumor cells can and lead to their death (through either apoptotic or necrotic activity) as well as by induction of aging (Carvalho et al., 2011; Dudkowska and Kucharewicz, 2013).

In the antiangiogenic therapy, many compounds also found in natural products such as fruits, vegetables, spices, and green tea were used. Many compounds present in natural products such as fruits, vegetables, spices, and green tea are also being used in the antiangiogenic therapy. Medicinal substances are present in the whole plants or in their particular organs (stems, leaves, roots, seeds). The most common plants in discussed area are *Selaginella tamariscina* Beauv (Hsin et al., 2013), *Gleditsia sinensis* (Yi et al., 2012), *Acer tegmentosum* (Kim et al., 2015), *Viscum album* (Taraphdar et al., 2001), *Strychnos nux-vomica* (Lee et al., 2008b), *Apium graveolens* (Shukla and Gupta, 2010), *Rosmarinus officinalis* (Tai et al., 2012), *Brucea javanica*, and *Hypericum perforatum* (Agostinis et al., 2002; Lau et al., 2008). These and many other plants contain substances of a therapeutic use, including

polyphenols and their derivatives (which are the most important group), anthracycline antibiotics and their quinone analogs, alkaloids, and other metabolites of the integrated nitrogen atom in the molecule, terpenoids, polysaccharides, polyamines, and cytokines (Batra and Sharma, 2013; Dudkowska and Kucharewicz, 2013; Najda et al., 2014; Taraphdar et al., 2001).

As a figure 3 shows chemical structure with the extracted from medicinal plants that directly inhibit angiogenesis.

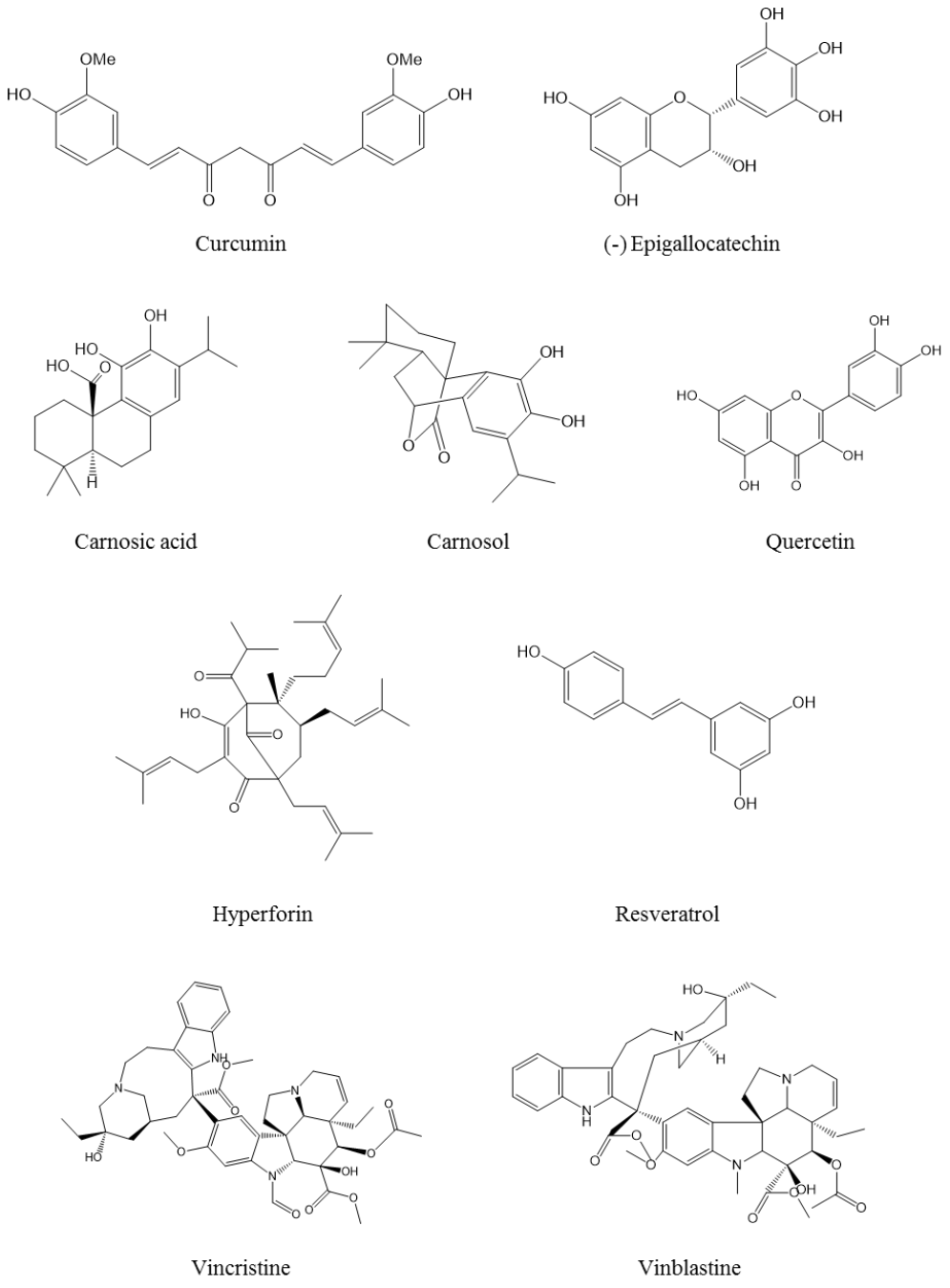


Figure 3. Bioactive compound known as an anti-angiogenics

5. Anti-angiogenic natural products and ocular neovascular diseases

Unstable and leaky vasculature is formed during rapid uncontrolled ocular angiogenesis. This leads to hemorrhage and accumulation of fluids and protein exudates in ocular cavities, causing reduction in the transparency of the cornea and impairment of the structure and function of retinal neurons resulting in vision loss. These vessels may induce the formation of fibrous scarring, causing irreversible damage to retinal function that can eventually result in blindness if left untreated (Zhang and Ma, 2007).

In a kind of compound, a part of natural compounds has been tested for their effects in ocular neovascular diseases specifically, and some have very promising activity. Polyphenols are the most abundant secondary metabolites, constituting the active substances in many medicinal plants. Polyphenols have long been recognized for their antioxidant properties (Manach et al., 2004). Polyphenolsvare loosely defined as having several hydroxyl groups on aromatic rings. They are divided into classes such as phenolic acids, flavonoids, stilbenoids and lignans, according to the number of phenolic groups and the structures that connect these rings to one another (Manach et al., 2004). The flavonoids are the most common class of polyphenolic compounds that are found ubiquitously in plants. They share a common structure of two aromatic rings that are connected together by three carbon atoms that form an oxygenated heterocycle (Manach et al., 2004). They are divided into subclasses according to the substitutions on the heterocycle and the position and length of the

linker between the cyclic moieties, and include flavonols (e.g., quercetin), flavones (e.g., luteolin and apigenin), isoflavones (e.g., genistein), flavanones (e.g., hesperetin) and homoisoflavanones (e.g., cremastranone) (Figure 4). Many flavonoids have been studied for their beneficial roles in ocular diseases (Majumdar and Srirangam, 2010). Medicinal plants continue to provide new sources of compounds for the treatment of disease. Natural products that have been shown to reduce angiogenesis provide an appealing alternative to the available biologic pharmacotherapies for ocular neovascular diseases, and hopefully more of these compounds will be tested in the ocular context, drawing on the wealth of compounds that show antiangiogenic activity in cancer models (Sagar et al., 2006a, b).

.

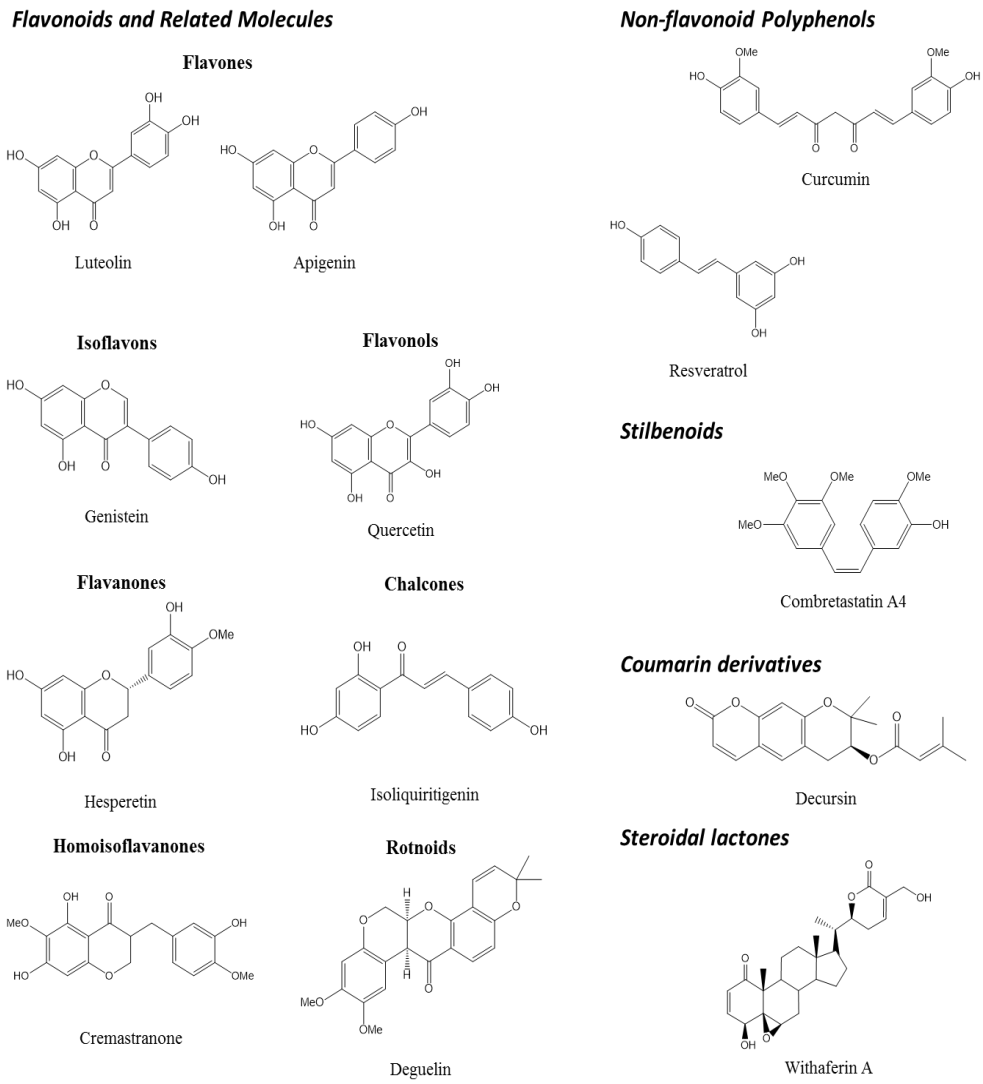


Figure 4. Chemical structure of anti-angiogeic natural products tested in ocular neovascular diseases

6. The purpose of this study

Based on the importance of identifying bioactive natural compounds in anti-angiogenic activity, the present study attempts to discover novel mechanisms of action of wondonin analogues.

Wondonin, a natural product from *Poecillastra wondoensis* and *Jasspis* sp. marine sponge, is a new structure to imidazole alkaloids containing a bis(dihyroxystyrene) moiety; this alkaloid has been reported to be a potent anti-angiogenic compound (Jun et al., 2007; Shin et al., 2001). However, wondonin is difficult to obtain large quantities and don't know accurate mechanism about anti-angiogenesis. Thus, synthesized many kinds of analogues about wondonin and investigated to anti-angiogenic effect.

In the present study, targets that are more relevant for the anti-angiogenic activity of wondonin analogues were investigated using human umbilical vein endothelial cells (HUVEC) and mouse embryonic stem cell (mES). In addition, a novel mechanism of action for wondonin analogues was investigated about pathogenic angiogenesis like diabetic retinopathy.

Thus, these wondonin analogues may potential as therapeutic agents to disease caused by excessive angiogenesis.

II. Mechanism for Anti-angiogenic Activity of Wondonin Analogues

1. Introduction

Angiogenesis is the formation of new blood vessels from pre-existing ones. This acquisition of blood capillary is also able to be detected under normal physiological conditions such as embryonic vascular development and wound healing. However, an excessive angiogenesis is highly associated with the disease processes including diabetic ocular neovascularization, duodenal ulcers, arthritis, and cancer (Folkman, 1995).

Vascular endothelial growth factor (VEGF) is one of the most important factors regulating angiogenesis. VEGF, enriched in the host cell tissue for new blood vessel, plays a role in vascular permeability, growth of vascular networks, and survival of vessel cells (Ferrara and Bunting, 1996). Therefore, the modulation of VEGF-mediated angiogenic pathway is considered an important target in the drug discovery program for the diseases associated with the over-activation of angiogenesis.

In general, the activity of VEGF is mediated by VEGF receptors (VEGFR)-1 (Flt-1) and VEGFR-2 (KDR/Flk-1) in endothelial cells (Abdullah and Perez-Soler, 2012). In particular, VEGFR-2 is implicated in all aspects of normal and pathological vascular endothelial cell processes. VEGFR-2 is also the key mediator of endothelial cell survival, migration and proliferation. In addition, VEGF/VEGFR-2 axis is also associated with the permeability of blood vessel (Miller et al., 2013; Zhang et al., 2016). Therefore, the overexpression of VEGF is able to activate several VEGF-mediated signaling pathways in endothelial cells, leading to induction of an abnormal extent and various malfunctions of physiological processes.

Recent findings also suggest that diabetic retinopathy (DR), one of intraocular vascular disease, age-related macular degeneration (AMD), and retinal vein occulusion (RVO), are all caused by iris neovascularization (Miller et al., 2013). Especially, the increased levels of VEGF were found in DR patients, and VEGF was also upregulated in ischemia retina by intraocular neovascularization (Adamis et al., 1994; Ashton et al., 1954; Grossniklaus et al., 2010; Shima et al., 1996). In addition, the injection of recombination human $VEGF_{165}$ also induced intraocular neovascularization (Tolentino et al., 1996). These events strongly suggest that VEGF is the key factor in the intraocular vascular disease. Therefore, the small molecule inhibitors of VEFG/VEGFR-2 mediated signaling pathways might be applicable in a variety of neoangiogenesis-associated pathophysiological diseases.

In this study, a series of wondonin analogues were designed, synthesized, and evaluated. Our efforts have resulted in drug-like analogues with more potent anti-angiogenic activity.

B. Materials and Methods

1. Materials

1.1. Reagents

Dulbecco's modified Eagle medium (DMEM), fetal bovine serum (FBS), antibiotic-antimycotic solution, trypsin-EDTA, TRI reagent, Alexa Fluor 488-labeled chicken anti-rat IgG was purchased from Molecular Probes (Invitrogen, Carlsbad, CA). 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), dimethyl sulfoxide (DMSO), gelatin from Sigma–Aldrich (St. Louis, MO). Recombinant human VEGF ($VEGF_{165}$) was obtained from R&D Systems (Minneapolis, MN). Growth factor-reduced Matrigel was purchased from BD Biosciences (San Jose, CA). Mouse anti-phospho-ERK1/2, anti-β-actin, were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Rabbit anti-HIF1a, anti-VEGFR2, anti-phospho-VEGFR2, anti-phospho-SAPK/JNK (Thr^{183}/Tyr^{185}), anti-phospho-p38, anti-PI3K, anti-phospho-PI3K, anti-AKT, anti-phospho-AKT(Ser^{473}/Thr^{308}), anti-mTOR (Ser^{2448}), anti-p70S6K (Thr^{389}), anti-eNOS, anti-phospho-eNOS anti-Src, anti-phospho-Src, anti-phospho-FAK were purchased from Cell signaling Technology (Beverly, MA). Gene-specific primers for real-time PCR were synthesized from Bioneer Corporation (Daejeon, Korea). Reverse Transcription Kit was purchased from Promega (Madison, WI).

1.2. Compounds

Wondonin was isolated from *Poecillastra wondoensis* and *Jasspis* sp. marine sponge, as decribed previously. (Shin et al., 2001) Although the biological activities of wondonin is promising, the abundance of wondonin in marine natural products is very low. Therefore, very efficient methods for the synthesis of wondonin analogues were developed by research collaborator and the synthetic compounds were kindly provided.

1.3. Cell Culture

Human umbilical vascular endothelial cells (HUVEC) was obtained from ATCC (Rockville, MD) and cultured in EGM-2 (Lonza, Walkerswille, MD) supplemented with 10% FBS at 37 ℃ in a 5% CO_2 atmosphere. To induce hypoxia, cells were incubated in the hypoxia chamber (Billups-Rothenberg, San Diego, CA) with a mixture of 1% O_2, 5% CO_2, and 94% N_2.

2. Methods

2.1. Cell growth assay

Cell growth was assessed by an MTT assay. HUVECs (8×10^3 cells/well) were seeded into a 96-well plate with EGM-2 medium supplemented with 10% FBS for 24 h. Next day, the culture medium was removed, and then incubated with serum-free medium for 12 h. Following serum starvation, the cells were cultured in fresh 2% FBS medium containing various concentrations of the test compound for 24 h in the presence of VEGF (50 ng/ml). In hypoxic condition (1% O_2), the cell cultured in fresh 5% FBS medium containing various concentrations of test compound at 37 ℃ for 24 h. After the incubation, an MTT solution was added, and the plate was incubated for an additional 4 h. The formazan product was dissolved in DMSO, and the absorbance was detected at 570 nm using VersaMax ELISA microplate reader (Molecular Devices, Sunnyvale, CA).

2.2. Scratch-wound migration assay

HUVECs were allowed to grow at 90% confluence in 12-well plates pre-coated with 0.2% gelatin and then incubated for 24 h. After cells were attached, the cells were wounded by scratching with a 0.2-ml pipette tip. Plates were then washed with serum-free medium. Fresh medium was replaced with 2% FBS/EGM-2 medium containing various concentrations of the test compound. The cells were incubated

for 24 h in normal condition or hypoxia chamber and the images were taken with an inverted phase contrast light microscope (Olympus Optical Co. Ltd., Tokyo, Japan).

2.3. *In vitro* capillary tube formation assay

Matrigel (70 μl/well) was coated on 96-well plate and polymerized for 30 min at 37 ℃ in a 5% CO_2 atmosphere. HUVECs (1.5×10^4 cells/well) and various concentration of compound were seeded onto each well of the Matrigel-coated 96-well plate. The cells were then incubated for 8 h at 37 ℃ in a 5% CO_2 atmosphere or hypoxia chamber. The formation of endothelial cell tubular structure was visualized under an inverted microscope and photographed (Olympus Optical Co. Ltd., Tokyo, Japan). Furthermore, tube formation was quantified by calculating the tube number and was expressed as a percentage by normalization with untreated control cells.

2.4. Transwell invasion assay

The HUVEC invasiveness was determined using a modified Boyden chamber model (Transwell apparatus, 8-μm pore size; Corning Inc., Corning, NY). Briefly, the upper side of chamber was coated with matrigel:PBS (1:30), and the lower side of filter was coated with 0.2 % gelatin for 30 min. After fresh EBM with 5% FBS was placed in the lower chamber and the HUVECs (4×10^4 cells/well) were seeded on diluted matrigel. Then, the cell was treated with test compound for 24 h in hypoxia chamber. After the incubation, the non-migrated cells on the upper side of chamber

were gently scraped away with a cotton swab. The cells were fixed with 4% paraformaldehyde for 10 min and stained with 1% crystal violet solution (sigma). Image were recorded using an OLYMPUS inverted microscope, and the migrated cells were quantified by Image J program. The percentage of migrated cells inhibited by test compound was normalized to the untreated control cell migration.

2.5. Real-time quantitative PCR

Total RNA was extracted from the HUVEC using TRIzol reagent (Invitrogen) and using the Revese Transcription System (Promega, MI, USA) according to the manufacturer's instructions. Real-time PCR was conducted using iQ SYBR Green Supermix (Bio-Rad), according to the manufacturer's instructions. The thermos cycling conditions utilized were 95 ℃ for 5 min prior to the first cycle; 40 cycle of 95 ℃ for 20 s, 56 ℃ for 20 s, and 72 ℃ for 30 s; 95 ℃ for 1 min; and 55 ℃ for 1min. The threshold cycle (C_T), indicating the fractional cycle number at which the amount of amplified target gene in each well reaches a fixed threshold was determined using MJ Research Opticon Monitor Software package (Bio-Rad, Hercules, CA, USA). Relative quantification, representing the difference between a sample treated group and the untreated control group, was calculated by the comparative C_T method as previously described (Livak and Schmittgen, 2001). The data were normalized to the housekeeping gene β-actin and fold changes in gene expression were analyzed using the equation $2^{-\Delta\Delta C_T}$, where $\Delta\Delta C_T$ = [C_T of target gene - C_T of housekeeping gene]$_{\text{treated group}}$ - [C_T of target gene - C_T of housekeeping

gene]$_{\text{untreated control group}}$.

2.6. Western blot analysis

The harvested cells were lysed through 2X sample loading buffer (250 mM Tris-HCl pH 6.8, 4% SDS, 10% glycerol, 0.006% bromophenol blue, 2% β-mercaptoethanol, 50 mM sodium fluoride and 5 mM sodium orthovanadate). The protein concentration was measured, and equal amounts of protein samples were subjected to 6-15% SDS-PAGE. The separated proteins were transferred to PVDF membranes (Millipore, Bedford, MA), which were then incubated with primary antibodies diluted in 2.5% BSA in TBST (1:500-1:1000) overnight at 4 °C. The membranes were then washed three times with TBST and incubated with the corresponding secondary antibodies. Protein bands were detected with an enhanced chemiluminescence detection kit (Intron, Daejon, Korea) and a LAS-4000 Imager (Fuji Film Corp., Tokyo, Japan).

2.7. *Ex-vivo* aortic ring assay

The mouse aortic ring assay was performed as previously described (Baker et al., 2012). Briefly, 48-well plates were coated with 150 μl of Matrigel and then incubated at 37 ℃ and 5 % CO_2 for 30 min. The aortas isolated from mice (Central Laboratory Animal Inc., Seoul) were removed of periadventitial fat, connective tissues and cut into 1-1.5 mm long rings. After rinsing with PBS, the aortas were placed in the

Matrigel coated wells and covered with an additional 100 μl of Matrigel. The artery rings were cultured in 1 ml of EGM with 5% FBS for 2 days and then the medium was replaced with 1ml of EGM containing 5% FBS with ST-XII-145 (0-2.5 μM). The medium was replaced every 2 days with medium that had the same composition as described above. After 6 days, the capillary vessel growth was measured by taking photographs with the OLYMPUS inverted microscope. The area of the capillary was estimated using an image J program.

2.8. Endogenous Alkaline phosphatase staining

Briefly, the embryos were fixed with fixation buffer (4% paraformaldehyde + 10% Triton-X100) at room temperature. After brief rinse with rinse buffer (10X PBS + 10% Triton-X100 + BSA + DDW) and staining buffer (5M NaCl, 1M MgCl2, 1M Tris, pH 9.5, 10% Tween-20), the embryos were developed with NBT/BCIP ready to use tablets (Roche, Mannheim, Germany) and developed until desired signal was obtained.

2.9. Zebrafish subintestinal vessel angiogenesis assay

The zebrafish embryos were maintained in a 12-well plate with egg water. At 24 hpf the embryos were removed the chorion by forceps and immersed the dechorionated embryos in a solution containing 0.003% 1-phenyl-2-thiourea in egg water to prevent pigmentation. And then, zebrafish embryos treated with various

concentrations of test compound. At 96 hpf the embryos were fixed with 4 % paraformaldehyde at room temperature. They were washed with PBS and photographed using fluorescence microscope (Axiovert 200M, Carl Zeiss, DE)

2.10. Zebrafish hyaloid vessel assay

Transgenic zebrafish (flk:EGFP) embryos were provided by the Korea zebrafish oraganogenesis muatant bank (ZOMB, Daegu, Korea). The zebrafish hyaloid vessel assay was performed as previously described (Jung et al., 2016). Zebrafish embryos (3dpf) were placed in a 12-well plate with 130 mM glucose embryonic water and various concentrations of test compound. At 6dpf, zebrafish larvae were fixed by 4% paraformaldehyde and stored overnight at 4 ℃. After then, larvae were washed by distilled water and incubated with 10X trypsin (2.5%) for 90 min at 37 ℃ in a 5% CO_2 atmosphere. They were washed with distilled water and then isolated lenses from zebrafish larvae. Optic disc (OD) branches were visualized and photographed using fluorescence microscope (Axiovert 200M, Carl Zeiss, DE). The diameters of vessel larvae were measured by ImageJ software.

2.11. Statistical Analysis

The data are expressed as the means ± SD of the indicated number of independently performed experiments. Statistical significance ($p < 0.05$) was assessed using Student's t-test for paired data.

Table 2. Sequences of target gene-specific primers used in the real-time PCR

Target genes		Sequences
HIF-1α	Sense	5’-GAT AGC AAG ACT TTC CTC AGT CG-3’
	Antisense	5’-TGG CTC ATA TCC CAT CAA TTC-3’
VEGF	Sense	5’-GAG CCT TGC CTT GCT GCT CTA C-3’
	Antisense	5’-CAC CAG GGT CTC GAT TGG ATG-3’
β-Actin	Sense	5’-AGC ACA ATG AAG ATC AAG AT-3’
	Antisense	5’-TGT AAC GCA ACT AAG TCA TA-3’

C. Results

1. The selection of wondonin analogues I

The primary aim for this study was to discover a new scaffold from the structure of wondonins that are chemically stable and easily accessible with the drug-like properties.

To investigate the selection analogue of the anti-angiogenesis activity in HUVECs, selectivity index (SI) with the ratio of IC_{50} value for the tube formation to IC_{50} value for the cell growth were primarily employed.

First, wondonin analogues SI value was compared with wondonin SI value (> 1.7) and then selected analogues higher than wondonin. However, SI didn't present a great contrast to each analogues.

Next, Tube IC_{50} used to measure the selection of analogues. Among the many analogues, anti-angiogenic activity of ST-XII-145 was higher than any other analogues. Especially, ST-XII-145 was remarkable activity when measured against wondonin. So, ST-XII-145 among all analogues was chosen and it was investigated about anti-angiogenic activity.

Table 3. Inhibition of VEGF-induced tube formation and cytotoxicity I

No.	Sample	Tube IC_{50} (μM)	MTT IC_{50} (μM)	SI (MTT IC_{50}/ Tube IC_{50})	Wondonin vs activity
1	ST-VII-183	23.74	>100	>8.4	1.8
2	ST-IX-122	27.97	95.87	3.43	1.53
3	ST-IX-136	>40	>100	-	-
4	SI-I_005	15.86	>100	11.3	2.7
5	SI-I-007	>40	>100	-	-
6	SI-I-008	9.96	37.2	3.73	4.29
7	SI-I-009	>20	45.91	-	-
8	SI-I-011	>20	>100	-	-
9	SI-I-015	32.32	>100	8.37	1.32
10	ST-X-25	6.1	22.04	3.61	7.01
11	ST-X-26A	19.66	91.7	4.67	2.17
12	ST-X-26B	19.19	48.91	2.55	2.23
13	ST-X-31	26.44	32.54	1.23	1.62
14	ST-X-32	10.98	>100	10.57	3.89
15	ST-XI-139	6.92	33.48	4.84	6.18
16	ST-XI-134	4.21	19.95	4.74	10.15
17	ST-XI-130	3.24	22.58	6.98	13.21
18	ST-XI-133	6.73	20.81	3.09	6.35
19	ST-XI-140	14.92	31.53	2.11	2.87
20	ST-XI-100	4.37	21.05	4.82	9.79
21	ST-XI-106	14.17	17.39	1.23	3.02
22	ST-X-87	>20	>100	-	-
23	ST-XII-88	>20	>100	-	-
24	ST-XII-89	12.7	>100	>9.4	3.37
25	ST-XII-90	>20	>100	-	-

Table 3. Continued

No.	Sample	Tube IC_{50} (μM)	MTT IC_{50} (μM)	SI (MTT IC_{50}/ Tube IC_{50})	Wondonin vs activity
26	ST-XII-91	>20	>100	-	-
27	ST-XII-92	>20	96.84	-	-
28	ST-XII-93	>20	65.37	-	-
29	ST-XII-94	11.48	46.64	4.06	3.72
30	ST-XII-95	>20	51.86	-	-
31	ST-XII-96	12.63	>100	>9.5	3.38
32	ST-XII-97	8.52	>100	>14.1	5.02
33	ST-XII-98	>20	>100	-	-
34	ST-XII-99	17.88	>100	>6.7	2.39
35	ST-XII-100	>20	>100	-	-
36	ST-XII-101	>20	>100	-	-
37	ST-XII-102	>20	>100	-	-
38	ST-XII-103	19.89	95.28	4.79	2.15
39	ST-XII-104	>20	>100	-	-
40	ST-XII-105	6.71	31.44	4.69	6.37
41	ST-XII-106	11.95	33.76	2.83	3.58
42	ST-XII-107	>20	71.33	-	-
43	ST-XII-108	17.6	36.82	2.09	2.43
44	ST-XII-109	4.56	59.17	12.98	9.38
45	ST-XII-110	>20	>100	-	-
46	ST-XII-111	>20	28.86	-	-
47	ST-XII-112	9.99	>100	>12	4.28
48	ST-XII-113	10.6	15.7	1.48	4.03
49	ST-XII-115	11.32	>100	>10.6	3.78
50	ST-XII-127	3.04	37.33	12.28	14.06

Table 3. Continued

No.	Sample	Tube IC_{50} (μM)	MTT IC_{50} (μM)	SI (MTT IC_{50}/ Tube IC_{50})	Wondonin vs activity
51	ST-XII-128	4.61	30.13	6.54	9.27
52	ST-XII-126	>20	>100	-	-
53	ST-XII-142	4.82	15.91	3.3	8.87
54	ST-XII-143	3.13	10.78	3.44	13.66
55	ST-XII-144	6.11	17.08	2.8	7
56	ST-XII-145	0.72	4.92	6.83	59.38
57	Iso-Wondonin	7.43	>40	>5.83	5.75
58	Wondonin	42.75	>100	>1.7	1

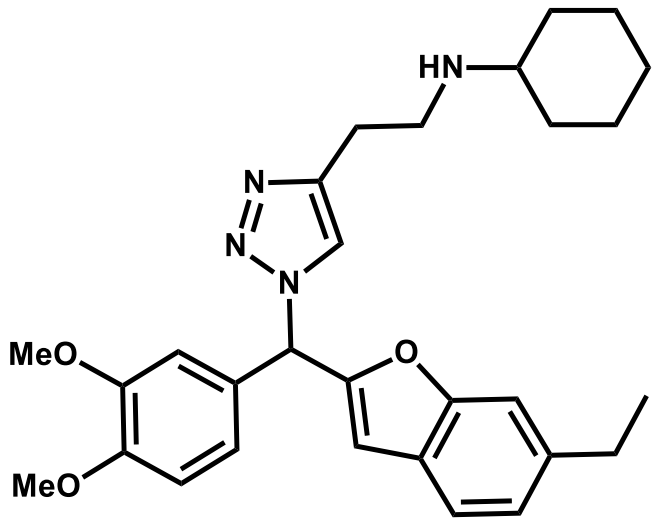


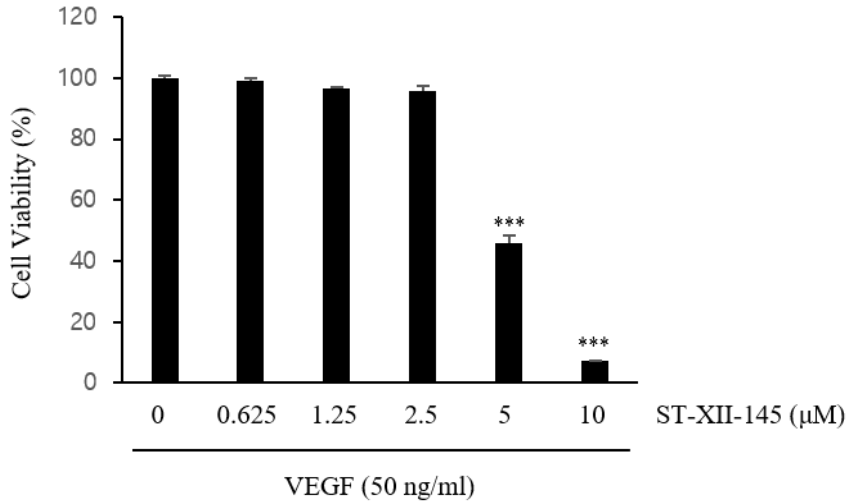
Figure 5. Chemical structure of ST-XII-145

2. Growth inhibitory activity of ST-XII-145 in HUVECs

The growth-inhibitory activities of the wondonin analogue ST-XII-145 against HUVECs was evaluated by MTT assay in VEGF-induced condition and hypoxia condition. As a shown in Figure 6, ST-XII-145 exhibited anti-proliferative activity against HUVECs in a concentration dependent manner, and the IC_{50} value were 4.92 μM (VEGF-induced condition) and 9.25 μM (hypoxia condition) for an incubation of 24 h.

Although the IC_{50} of ST-XII-145 in hypoxia condition was higher than IC_{50} of ST-XII-145 in VEGF-induced condition, overall condition with ST-XII-145 represented cytotoxicity in 5 μM or more concentration. Therefore, further analyses of the biological activities of ST-XII-145 were performed using up to a 2.5 μM concentration of ST-XII-145 in HUVECs.

A


120
100
80
60
40
20
0
Cell Viability (%)
***
***
0
0.625
1.25
2.5
5
10
ST-XII-145 (μM)
VEGF (50 ng/ml)

B

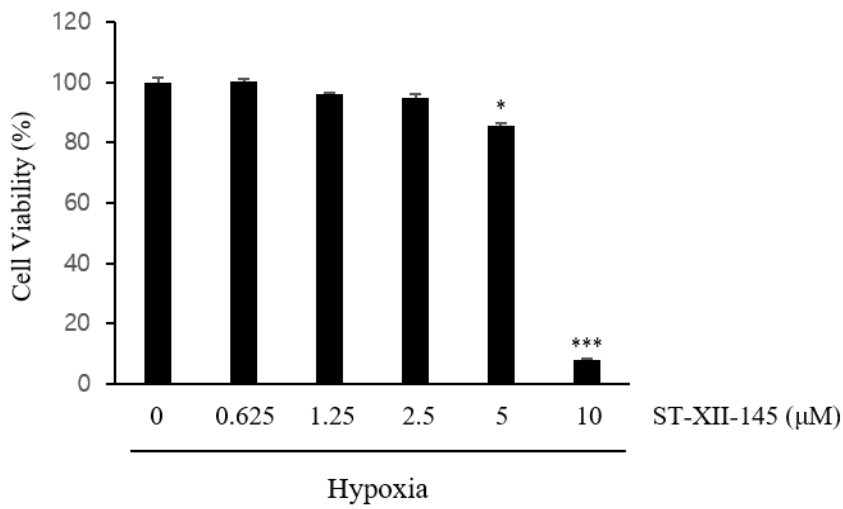

120
100
80
60
40
20
0
Cell Viability (%)
*
***
0
0.625
1.25
2.5
5
10
ST-XII-145 (μM)
Hypoxia

Figure 6. Effects of ST-XII-145 on cell viability

(A) The HUVEC were cultured with ST-XII-145 (0 – 10 μM) in the presence of VEGF (50 ng/ml) for 24 h. (B) The HUVEC were cultured with ST-XII-145 (0 – 10 μM) in hypoxia condition for 8 h. Cell viability is expressed as the percentage compared to the vehicle-treated culture and is expressed as the mean ± S.D. (*$p < 0.05$, ***$p < 0.001$).

3. ST-XII-145 inhibits the VEGF-induced cell migration and cell tube formation of HUVECs

To further in detail elucidate the biological activities and underlying mechanisms of actions the most potent compound ST-XII-145.

Endothelial cell migration and tube formation are essential steps in angiogenesis. Primarily, the effect of ST-XII-145 on the migration and capillary tube formation were evaluated in VEGF-induced HUVECs. The enhanced cell migration capacity by VEGF was effectively inhibited with the treatment of ST-XII-145 in a concentration-dependent manner. (Figure A, A-1)

The capillary-like network is considered to be an important hallmark in the process of angiogenesis in endothelial cells. In addition, VEGF is capable to induce the formation of tube structures with more remarkable, stable, and longer tube network (Tozer et al., 2005). To this end, the effect of ST-XII-145 on the tube formation of endothelial cells was also investigated in Matrigel. The findings showed that the VEGF-induced increased tubular formation was remarkably suppressed with the treatment of ST-XII-145 in a concentration-dependent manner. (Figure B, B-1)

In particular, the cell migration and tube formation were significantly inhibited by the treatment with 2.5 μM ST-XII-145 in VEGF-stimulated HUVECs, indicating the anti-angiogenic activity of ST-XII-145 is associated with the inhibition of endothelial cell migration and tube formation.

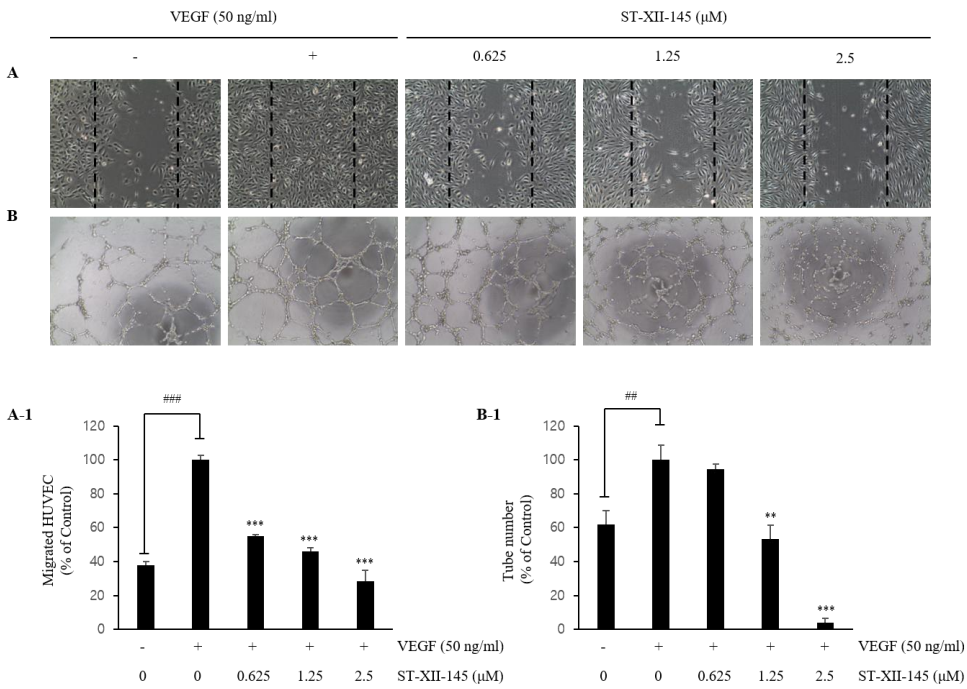

VEGF (50 ng/ml)
ST-XII-145 (μM)
-
+
0.625
1.25
2.5
A
B
A-1
B-1
Migrated HUVEC (% of Control)
Tube number (% of Control)
###
##
***
**
VEGF (50 ng/ml)
ST-XII-145 (μM)
0
0.625
1.25
2.5

Figure 7. The inhibitory effects ST-XII-145 on cell migration and capillary-like tube formation

(A, A-1) In cell migration assay, HUVECs were grown to confluence in gelatin-coated 12-well plates and then scratched with a tip. The cells were treated with VEGF (50 ng/ml) in the presence or absence of various concentration of ST-XII-145 for 24 h. The migrated cells were counted and compared to the vehicle-treated control groups. (B, B-1) In tube formation assay, HUVECs were seeded in Matrigel-coated 96-well plates. The cells were treated with VEGF (50 ng/ml) in presence or absence of various concentration of ST-XII-145. After incubation for 4-8 h, the capillary structures were photographed and quantified. The data were represented as the mean ± S.D. ($^{###}p < 0.001$; $*p < 0.05$, $***p < 0.001$).

4. ST-XII-145 inhibits cell migration, tube formation and invasion in hypoxic condition

Endothelial cell proliferation and *in vitro* migration were up-regulation by hypoxia condition (Michiels et al., 2000). To determine whether the effect of ST-XII-145 on endothelial cells, we evaluated the cell migration assay, capillary tube formation and invasion assay of the HUVEC under hypoxic conditions.

ST-XII-145 suppressed the movable ability of HUVEC in a concentration-dependent manner. (Figure 8A, A-1) Next, we investigated the effect of ST-XII-145 on tube formation of endothelial cells in Matrigel. Data showed that ST-XII-145 inhibited tubular network formation of the HUVEC in a dose-dependent manner. Remarkably the effective concentration was 2.5 μM (Figure 8B, B-1). In addition, invasive activity of HUVECs was determined by Matrigel coated transwell migration assay. The number of cells were found on the lower surface of membrane. When HUVECs were treated with ST-XII-145, fewer cells invaded to the transwell membrane (Figure 8C, C-1). Thus, ST-XII-145 may inhibit angiogenesis through repressing cell migration, tube formation and invasion.

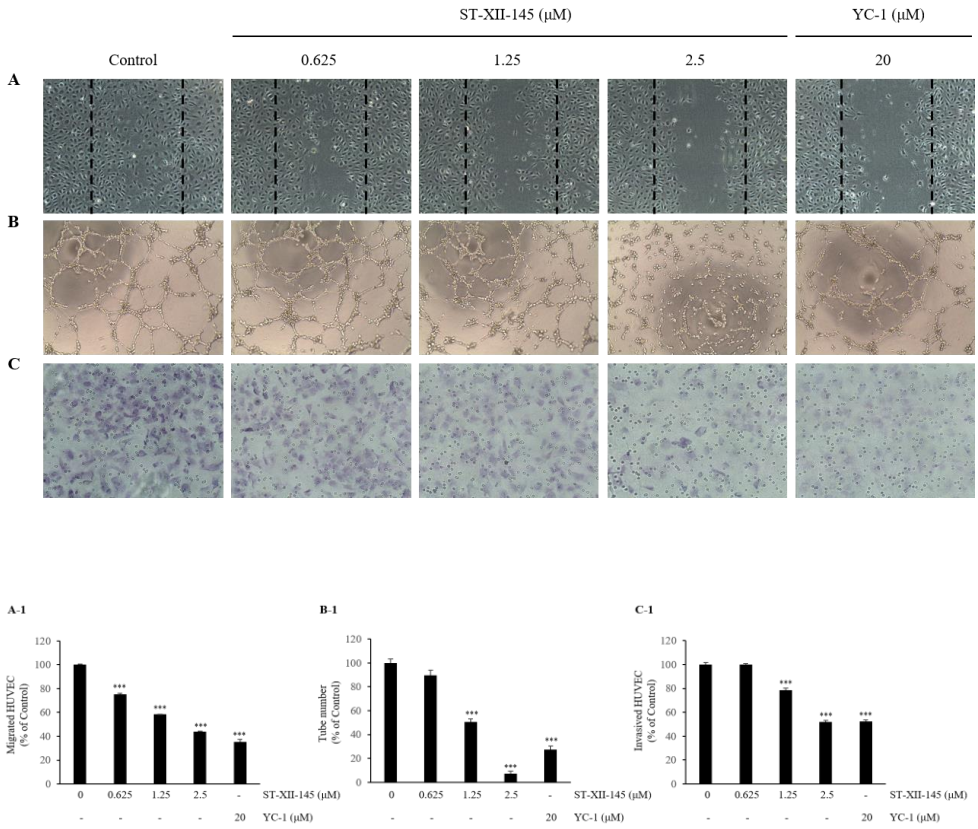

ST-XII-145 (μM)
YC-1 (μM)
Control
0.625
1.25
2.5
20
A
B
C
A-1
B-1
C-1
Migrated HUVEC (% of Control)
Tube number (% of Control)
Invasived HUVEC (% of Control)
0
20
40
60
80
100
120
***
0
0.625
1.25
2.5
-
ST-XII-145 (μM)
-
-
-
-
20
YC-1 (μM)

Figure 8. The inhibitory effects ST-XII-145 on cell migration, capillary-like tube formation and invasion assay

(A, A-1) In cell migration assay, HUVECs were grown to confluence in gelatin-coated 12-well plates and then scratched with a tip. The cells were treated with ST-XII-145 (0 – 2.5 μM) or YC-1 (20 μM) for 24 h in hypoxic condition. The migrated cells were counted and compared to the control group. (B, B-1) In tube formation assay, HUVECs were seeded in Matrigel-coated 96-well plates. The cells were treated with ST-XII-145 (0 – 2.5 μM) or YC-1 (20 μM). After incubation for 4-8 h, the capillary structures were photographed and quantified. (C, C-1) HUVEC treated with ST-XII-145 (0 – 2.5 μM) or YC-1 (20 μM) were seeded in the upper chamber, and then bottom chamber was filled with EBM medium containing 5 % FBS. The cells with an irregular shape in the images are cells that migrated into the lower chamber. The data were represented as the mean ± S.D. (***$p < 0.001$).

5. ST-XII-145 inhibits VEGF signaling pathway in VEGF-stimulated HUVECs

To further clarify the mechanisms of action underlying the anti-angiogenic activity of ST-XII-145, signaling proteins that are unconverted or inactivated in HUVECs were examined by western blot analysis.

VEGFR2 plays a major role in VEGF-dependent angiogenesis. Indeed, the VEGF-VEGFR2 axis signaling seems to be the most important pathway that executes the angiogenesis process by inducing proliferation, survival, migration, and sprouting of endothelial cells (Lohela et al., 2009). VEGFR-2 expression was increased by VEGF stimulation and then treatment with ST-XII-145 effectively decreased the levels of VEGFR-2.

Next examined the modulation of VEGF-stimulated MAPK signaling pathways in HUVECs. ST-XII-145 effectively suppressed the activation of p38 phosphorylation but doesn't affect about phosphorylation of ERK and JNK. ERK, one of the MAPK signaling pathways, has been implicated in the regulation of angiogenesis via various functions including cell proliferation, migration, and survival (Pages et al., 2000; Risau, 1997). JNK signaling is associated with cellular stress signals and initiation of apoptosis (Weston and Davis, 2002). On the other hand, p38 MAPK appeared to mediate migration by its ability to enhance actin polymerization (Rousseau et al., 2000a; Rousseau et al., 2000b). Furthermore, p38 MAPK has been shown to be a key regulator of vascular permeability induction by

VEGF (Clauss et al., 2001). These meaning that ST-XII-145 doesn't affect to cell survival but effect to cell permeability.

As a shown in Figure 9, ST-XII-145 effectively suppressed the activation of PI3K phosphorylation. In addition, the suppression of PI3K by ST-XII-145 subsequently led to a blockade of the activation of AKT and its downstream effectors eNOS. However, it doesn't effect to another downstream signaling like that mTOR and p70S6K. Moreover, ST-XII-145 downregulated the expressions of cell migration-related protein such as FAK.

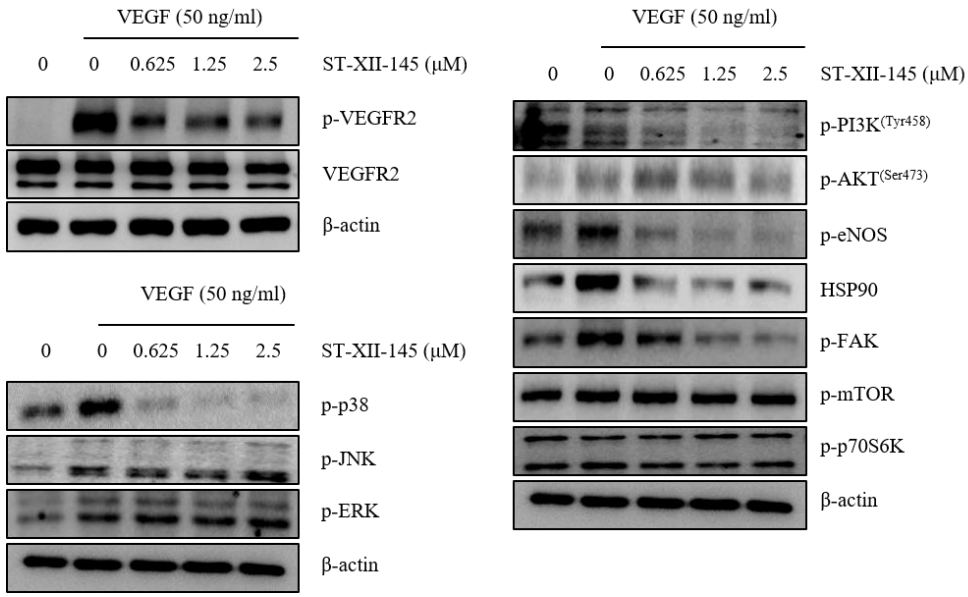


Figure 9. Effects of ST-XII-145 on VEGF signaling pathway in HUVEC

Western blot analysis of HUVECs. Cells were treated with various concentrations of ST-XII-145 for 30 min and then stimulated with VEGF (50 ng/ml) for 20 min. The protein expressions were determined by western blotting as described in Experimental Section. β-Actin was used as a loading control.

6. ST-XII-145 inhibits hypoxia-induced HIF-1α expression and HIF-1α signaling pathway

Hypoxia-induced VEGF secretion was regulated by HIF-1α (Hirota and Semenza, 2006). Furthermore hypoxia-induced VEGFR-2 protein expression was regulated by VEGF induced by HIF-1α (Tang et al., 2004).

In VEGF induced condition, ST-XII-145 regulated that expression of VEGFR-2 and PI3K-AKT-eNOS, p38, FAK signaling. Next investigated the effects of ST-XII-145 on HIF-1α, VEGF mRNA and protein expression by real-time PCR and western blot analysis. The result showed that hypoxia and ST-XII-145 had no effect on the HIF-1α mRNA expression. In contrast, the amount of HIF-1α protein increased in HUVECs under hypoxic conditions compare with that normoxia. Furthermore, ST-XII-145 inhibited hypoxia induced expression of HIF-1α protein.

In addition, hypoxia significantly increased the expression of VEGF mRNA and the hypoxia-induced VEGF mRNA expression was inhibited by ST-XII-145. Moreover, the amount of VEGF protein increased under hypoxic condition and VEGF protein level was decreased by ST-XII-145.

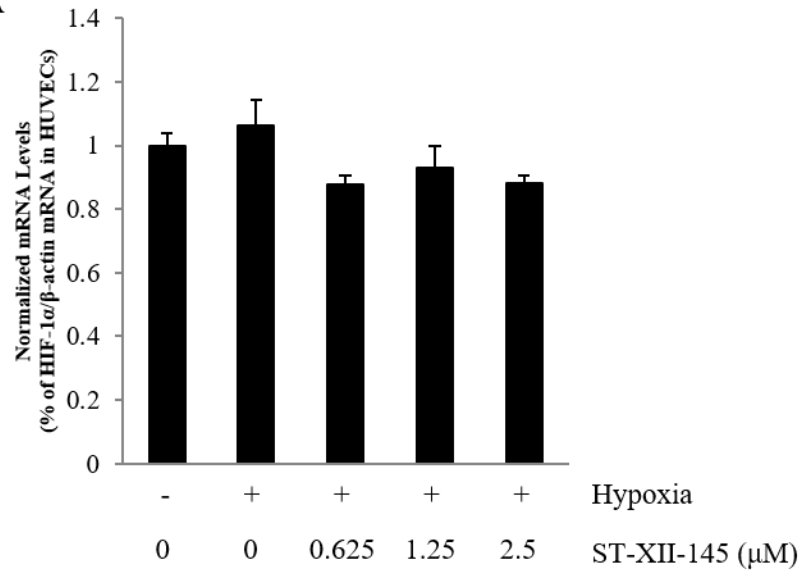
Next examined the modulation of MAPK signaling pathways in hypoxic condition. ST-XII-145 effectively inhibited the activation of p38 phosphorylation but doesn’t affect about phosphorylation of ERK.

Also, ST-XII-145 effectively inhibited the activation of AKT phosphorylation. In addition, the suppression of AKT by ST-XII-145 subsequently led to a blockade of

the activation of eNOS. Moreover, expressions of cell migration-related protein such as FAK were decreased by ST-XII-145.

These results suggest that the expression level of VEGF was regulated by HIF-1α protein. Especially, ST-XII-145 was a similar effect that both VEGF signaling pathway and HIF-1a signaling pathway.

A


Normalized mRNA Levels
(% of HIF-1α/β-actin mRNA in HUVECs)
1.4
1.2
1
0.8
0.6
0.4
0.2
0
-
+
+
+
+
Hypoxia
0
0
0.625
1.25
2.5
ST-XII-145 (μM)

B

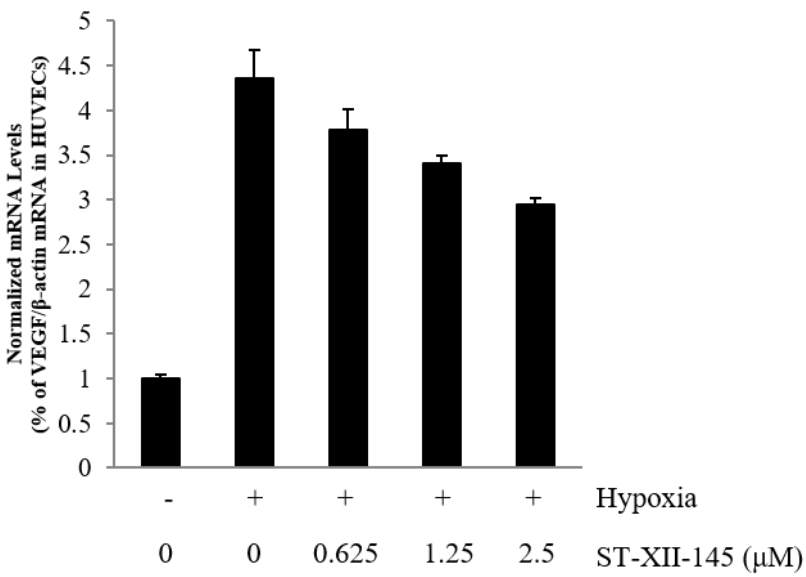

Normalized mRNA Levels
(% of VEGF/β-actin mRNA in HUVECs)
5
4.5
4
3.5
3
2.5
2
1.5
1
0.5
0
-
+
+
+
+
Hypoxia
0
0
0.625
1.25
2.5
ST-XII-145 (μM)

Figure 10. Effects of ST-XII-145 on the expression of HIF-1α and VEGF mRNA

Expression of HIF-1α and VEGF mRNA in HUVEC was examined by real-time PCR. Total RNA was isolated from cells cultured under normoxia or hypoxia with or without various concentration of ST-XII-145 for 4 h. The β-actin mRNA levels used for normalization.

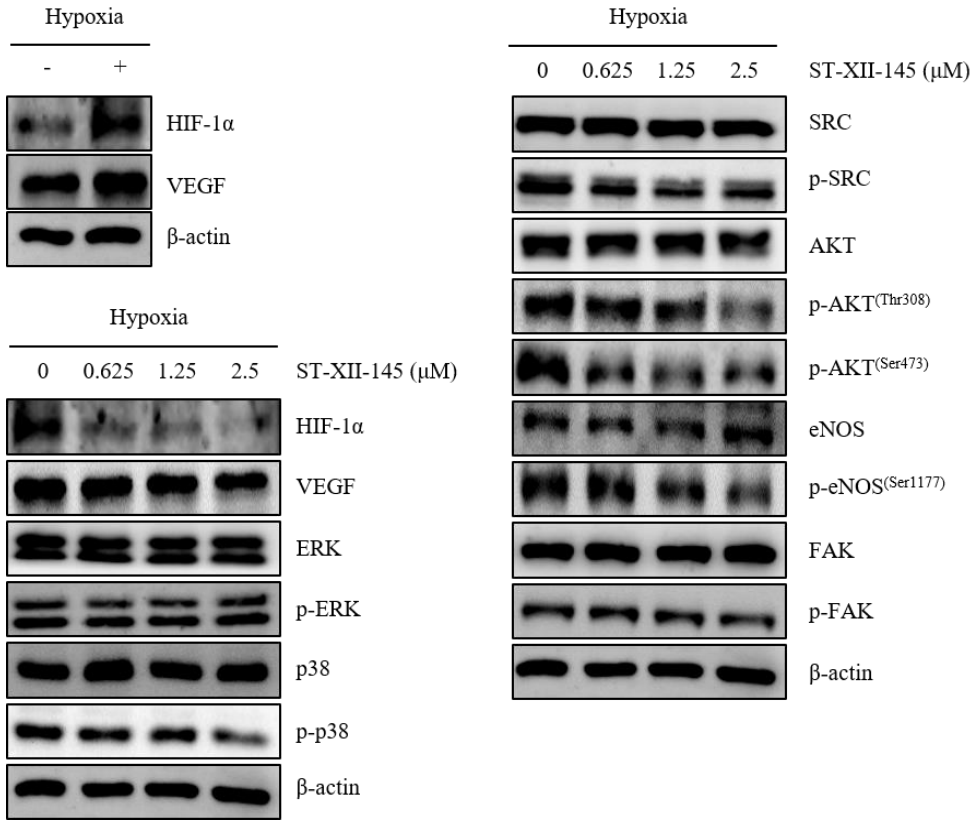
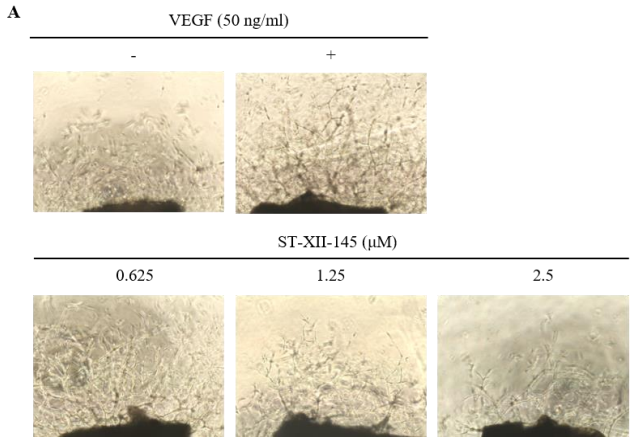


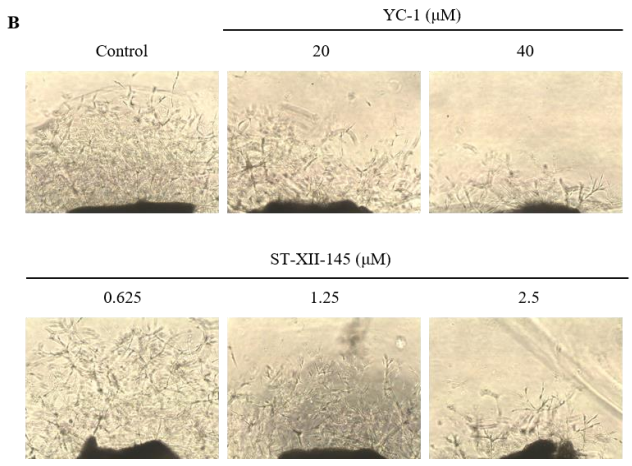
Figure 11. Effects of ST-XII-145 on HIF signaling pathway in HUVECs

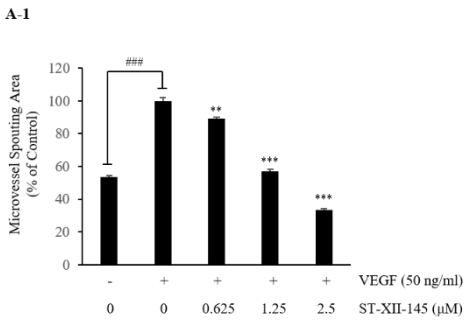
Western blot analysis of HUVECs. Cells were treated with various concentrations of ST-XII-145 in hypoxia condition for 8 h. The protein expressions were determined by western blotting as described in Experimental Section. β-Actin was used as a loading control.

7. ST-XII-145 inhibits capillary sprouting in the mouse aortic ring assay

Anti-angiogenic activities of ST-XII-145 were determined in an *ex vivo* angiogenesis model (Kruger et al., 2000) using the mouse aortic ring assay. This study used the aortas isolated from mice (Central Laboratory Animal Inc., Seoul) that cleaned of periadventitial fat, connective tissues and cut into 1-1.5mm long rings. Each rings were placed in the Matrigel, then 2-4 days after fibroblastic fusiform cells emerge from the ends of the aortic rings. The medium was changed every 2 days with vehicle or ST-XII-145 of various concentration. After 5-6 days, the ST-XII-145 treated aortic rings strongly inhibited the derivative of microvessel at 2.5 μM.


A
VEGF (50 ng/ml)
-
+
ST-XII-145 (μM)
0.625
1.25
2.5


B
YC-1 (μM)
Control
20
40
ST-XII-145 (μM)
0.625
1.25
2.5


A-1
Microvessel Spouting Area (% of Control)
###
**
***
***
VEGF (50 ng/ml)
ST-XII-145 (μM)

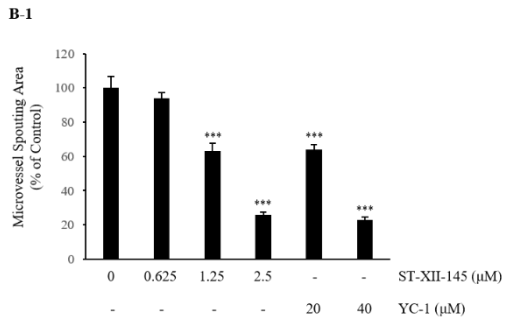

B-1
Microvessel Spouting Area (% of Control)
***
***
***
***
ST-XII-145 (μM)
YC-1 (μM)

Figure 12. Effects of ST-XII-145 on microvessel outgrowth arising from mouse aortic rings

The effect of ST-XII-145 on microvessel outgrowth arising from mouse aortic rings. Aortic rings isolated from mice were embedded in Matrigel in 48-well plates and fed medium containing various concentration of ST-XII-145 for 4 days in VEGF-induced and hypoxia condition. Representative photographs of three independent experiments are shown. The microvessel sprouting area was measured on day 4 of culture. The value are mean ± SD (n=3). ($^{###}p < 0.001$; $^{**}p < 0.01$, $^{***}p < 0.001$).

8. ST-XII-145 inhibits vasculogenic vessel in the zebrafish model

Zebrafish is useful *in-vivo* system for analyzing about angiogenesis. Many parts of zebrafish are similar to higher vertebrates. Such as circulatory system and the molecular mechanism of the developing blood vessel (Gore et al., 2012).

To investigated the anti-angiogenic activity of ST-XII-145 through a zebrafish angiogenesis model. Subintestinal vessel (SIV) formation that easily monitored in wild type embryos by the staining alkaline phosphatase activity and the transgenic Tg(*flk:egfp*) embryos.

First, we confirmed to through ALP assay model. To examine the effect of ST-XII-145, we treated to wild type zebrafish embryos at various concentration of ST-XII-145 during 72 hpf. At the result, ST-XII-145 inhibited neovascularization of the zebrafish SIVs in a dose-dependent manner. Next, we observed that anti-angiogenic effect of the ST-XII-145 comparison with wondonin and sunitinib in Tg(*flk:egfp*) embryo model. The effective concentration of the ST-XII-145 is 2.5 uM, and ST-XII-145 has more anti-neovascularization than wondonin and sunitinib. In these results, showing a strong indication that ST-XII-145 effectively inhibits neo-vascularization *in vivo*.

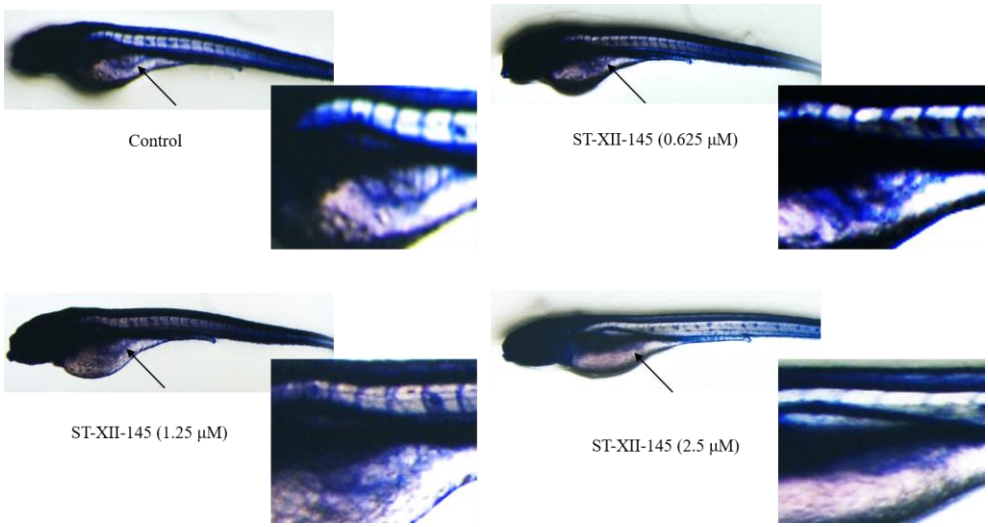


Figure 13. Effects of ST-XII-145 vasculogenic vessel formation from zebrafish (Alkaline phosphatase staining model)

Lateral view of AP stained embryos at 72 h pf. Representative images of AP-stained SIV basket of embryos treated with various concentration of ST-XII-145.

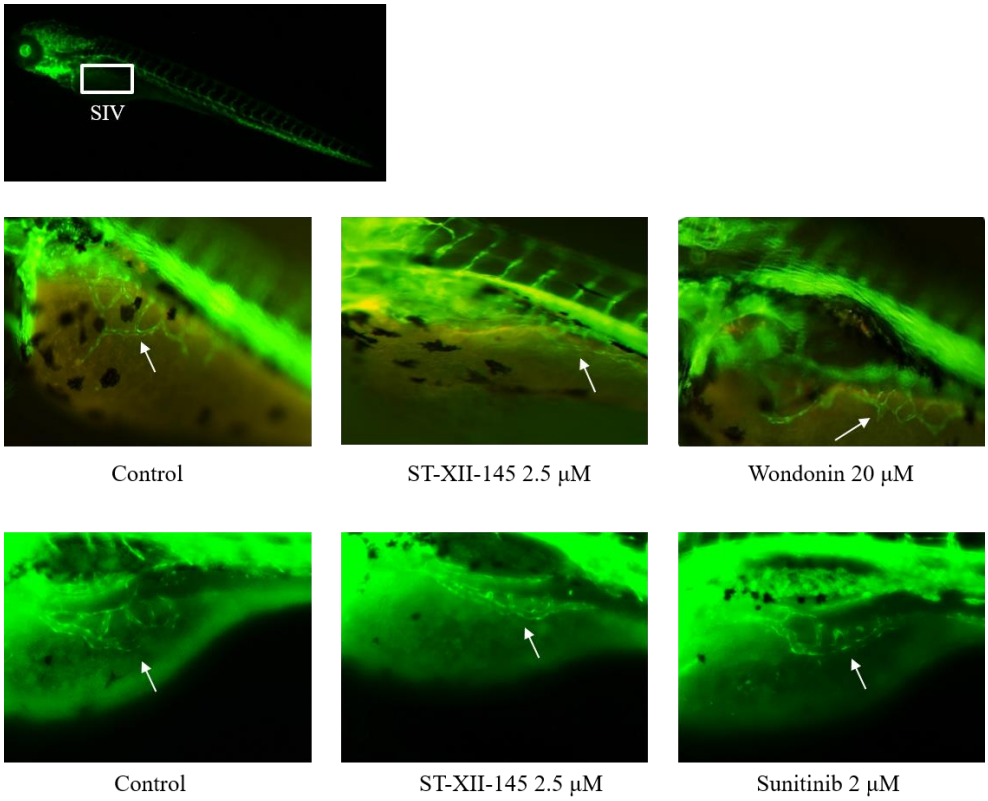


Figure 14. Effects of ST-XII-145 vasculogenic vessel formation from zebrafish (transgenic model)

Lateral view of EGFP-transgenic embryos at 72 h pf. Representative images of EGFP-transgenic SIV basket of embryos treated with ST-XII-145, wondonin and sunitinib.

9. Suppression of the expression of the endothelial biomarker PECAM in cultured EB-derived endothelial cells

To determine whether the ST-XII-145 mediated inhibition of endothelial-like cell growth was associated with the suppression of endothelial cell biomarker expression, the embryoid body (EB)-derived endothelial cells (on day 12) were treated with ST-XII-145 (0 – 2.5 μM) for 24 h. After treatment, the expression of the endothelial cell biomarker PECAM was also assessed in a 2-dimensional (2-D) culture. In addition, the protein expression of the endothelial cell-specific biomarker PECAM was analyzed. As shown in Figure16, PECAM was easily detected by immunofluorescence in the mES/EB-derived endothelial cells, but ST-XII-145 (2.5 μM) effectively suppressed PECAM expression.

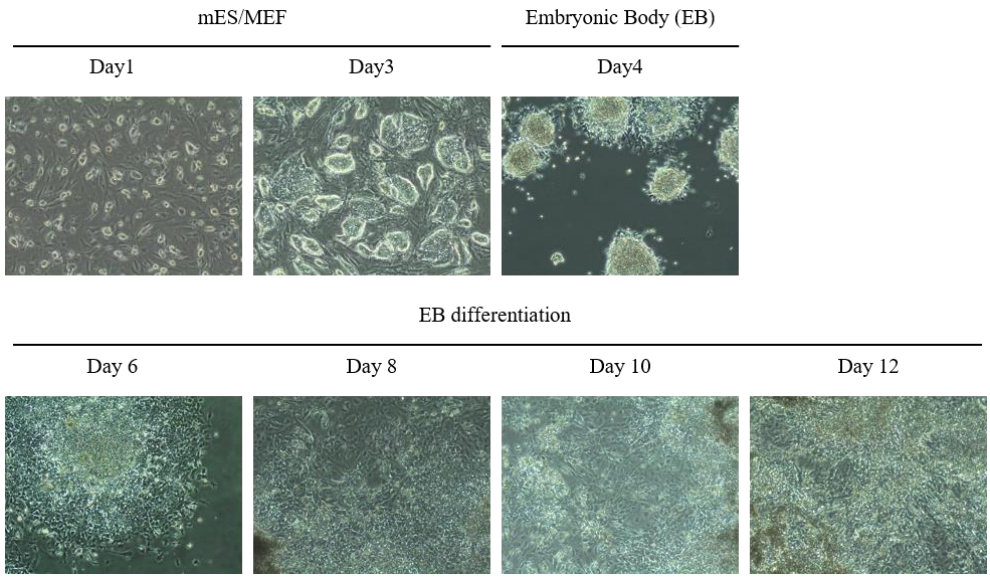


Figure 15. Differentiation of endothelial-like cells derived from mES/EBs

Morphology of differentiated mES grown in EGM-2 containing 5% FBS on gelatin-coated culture plates for 12 days.

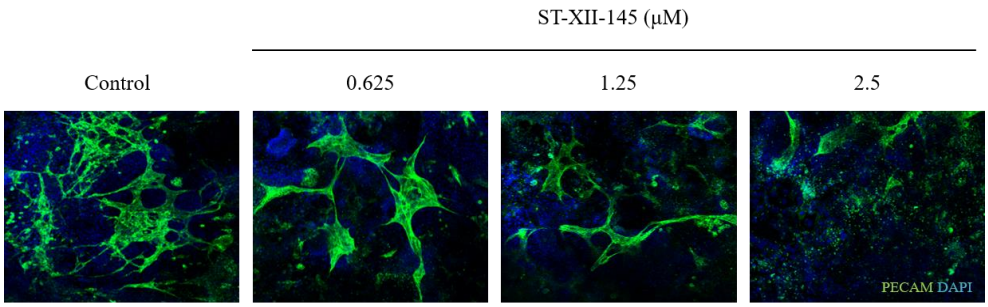


Figure 16. Effects of ST-XII-145 on vascularization of endothelial cells derived from mES/EBs

mES/EB-derived cells were differentiated for 12 d and then exposed to ST-XII-145 (0 – 2.5 mM) for 24 h. The mES/EB-derived endothelial cells were stained with an antibody directed against the endothelial cell biomarker PECAM. Nuclei were stained with DAPI

10. The selection of wondonin analogues II

With the purpose of improve of SI value, new analogues were synthesized that named LF. Total four kinds of LF were replaced ST-XII-145's benzofuran to benzothiazole.

To determine whether the SI value of LF analogues on HUVECs, about analogues evaluated the cytotoxicity and tube formation assay. As a result, SI value of all LF analogues was higher than ST-XII-145's SI value.

Among the LF analogues, SI value of LF-I-188 was highest and also anti-angiogenic activity was the same. So, LF-I-188 among all analogues was chosen, and then it is become new base analogue.

Table 4. Inhibition of VEGF-induced tube formation and cytotoxicity II

No.	Sample	Tube IC_{50} (μM)	MTT IC_{50} (μM)	SI (MTT IC_{50}/ Tube IC_{50})	Wondonin vs activity
1	LF-II-99	3.43	>40	>11.7	12.46
2	LF-II-100	>20	>40	-	-
3	LF-I-185	2.42	21.43	8.86	17.67
4	LF-I-188	1.39	38.59	27.76	30.76
5	Iso-Wondonin	7.43	>40	>5.83	5.75
6	Wondonin	42.75	>100	>1.7	1

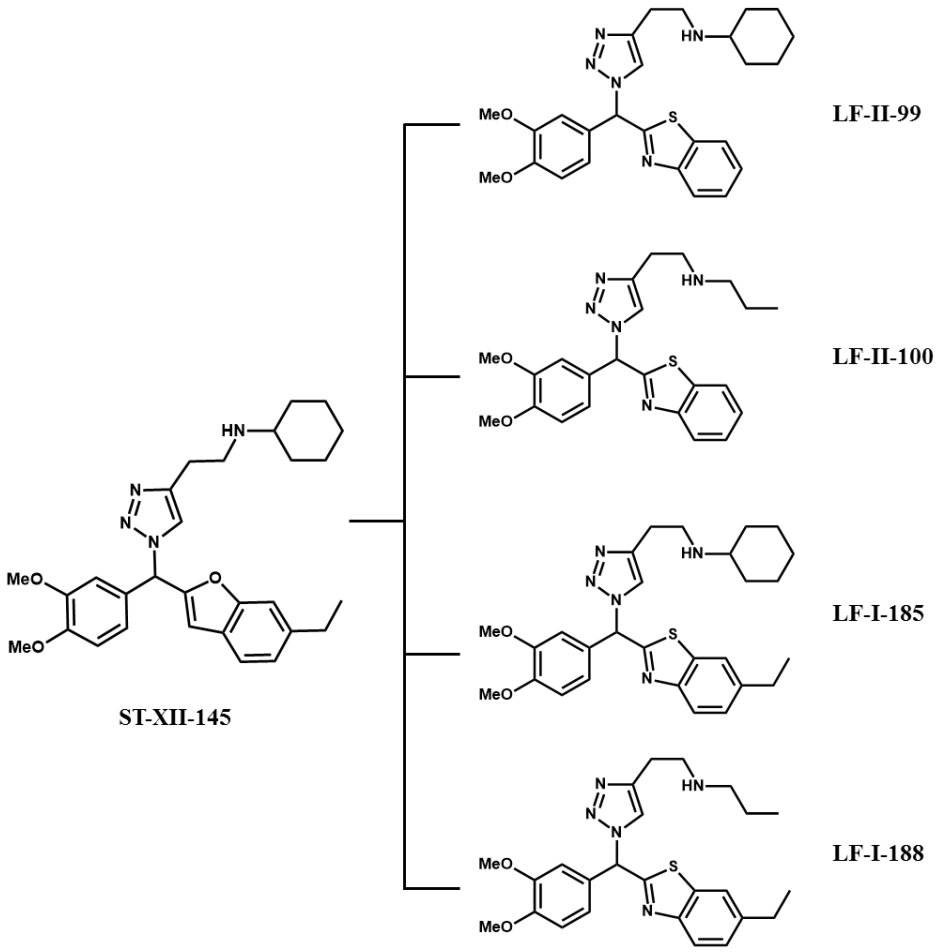


Figure 17. Chemical structure of LF analogues

11. The selection of wondonin analogues III

In order to more improve SI value and anti-angiogenic activity, total 15 kinds of new analogues synthesized. These new analogues were synthesized by base on previously analogue LF-I-188.

To determine whether the SI value of new 15 analogues on HUVECs, about analogues evaluated the cytotoxicity and tube formation assay. Consequently, ST2-VI-66 represented highest SI value and anti-angiogenic activity was similar to ST-XII-145.

Finally, ST2-VI-66 was selected that among synthesized all analogues until now. And then, to investigate anti-angiogenic effect about ST2-VI-66.

Table 5. Inhibition of VEGF-induced tube formation and cytotoxicity III

No.	Sample	MTT IC_{50} (μM)		Tube IC_{50} (μM)	SI (MTT IC_{50}/ Tube IC_{50})
		VEGF-induced	Growth medium	VEGF-induced	
1	ST2-VI-51	93.51	92.69	8.27	11.31
2	ST2-VI-60	>100	>100	4.73	>21.14
3	ST2-VI-63	70.31	66.04	2.84	24.76
4	ST2-VI-64	19.39	18.88	1.02	19.01
5	ST2-VI-65	>100	>100	3.93	>25.45
6	ST2-VI-66	47.45	48.59	0.63	75.32
7	ST2-VI-67	62.99	52.45	6.43	9.8
8	ST2-VI-68	>100	>100	2.12	>47.17
9	ST2-VI-69	85.2	90.12	13.58	6.27
10	LF-V-75	>100	>100	5.22	>19.16
11	LF-V-149	>100	>100	4.87	>20.53
12	LF-V-157	30.62	35.23	1.56	19.63
13	LF-V-165	75.59	74.71	3.4	22.23
14	LF-V-167	95.71	>100	1.53	62.56
15	LF-V-169	38.55	43.41	1.82	21.18
16	Sunitinib	9.92	>10	0.55	18.03

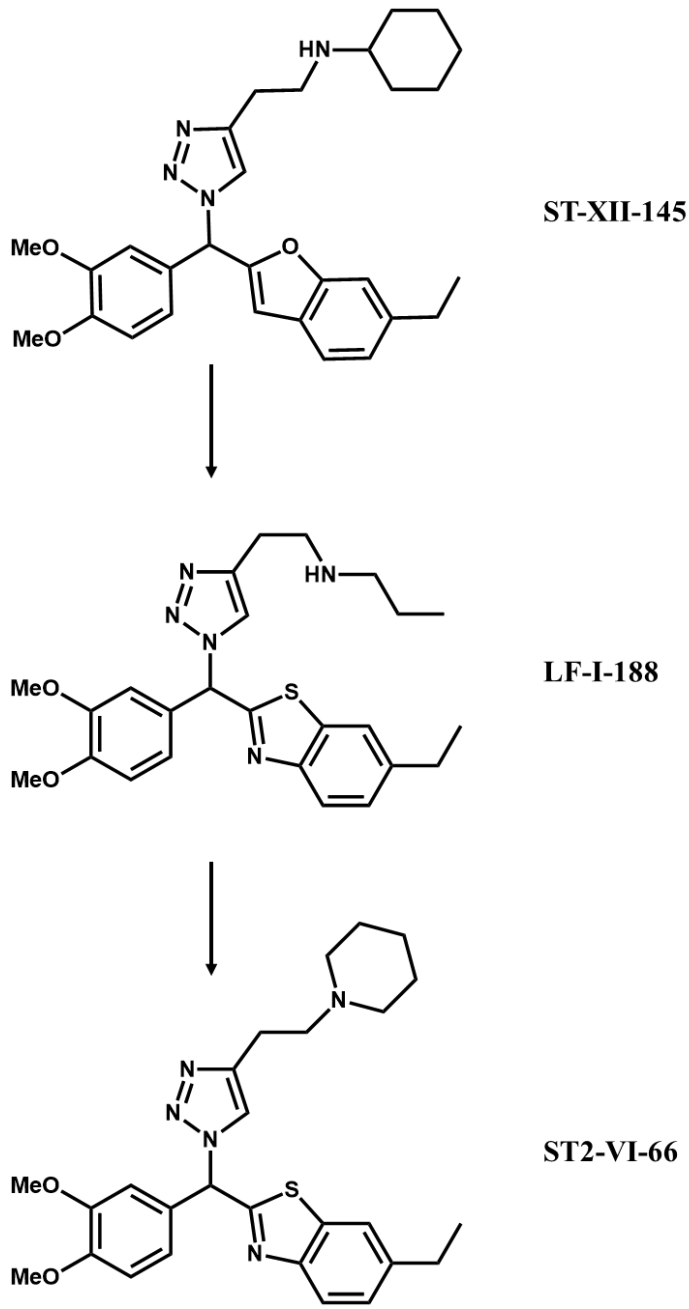


Figure 18. Chemical structure of ST2-VI-66

12. Effect of ST2-VI-66 on the migration and tube formation in VEGF-induced HUVECs

To further in detail elucidate the biological activities and underlying mechanisms of actions the most potent ST2-VI-66 was selected based on the highest selectivity index. Primarily, the effect of ST2-VI-66 on the migration and capillary tube formation were evaluated in VEGF-induced HUVECs.

The capillary-like network is considered to be an important hallmark in the process of angiogenesis in endothelial cells. In addition, VEGF is capable to induce the formation of tube structures with more remarkable, stable, and longer tube network (Tozer et al., 2005). To this end, the effect of ST2-VI-66 on the tube formation of endothelial cells was also investigated in Matrigel. The findings showed that the VEGF-induced increased tubular formation was remarkably suppressed with the treatment of ST2-VI-66 in a concentration-dependent manner (Figure 19A, A-1). The enhanced cell migration capacity by VEGF was effectively inhibited with the treatment of ST2-VI-66 in a concentration-dependent manner (Figure 19B, B-1).

In particular, the cell migration and tube formation were significantly inhibited by the treatment with 2.5 μM ST2-VI-66 in VEGF-stimulated HUVECs, indicating the anti-angiogenic activity of ST2-VI-66 is associated with the inhibition of endothelial cell migration and tube formation.

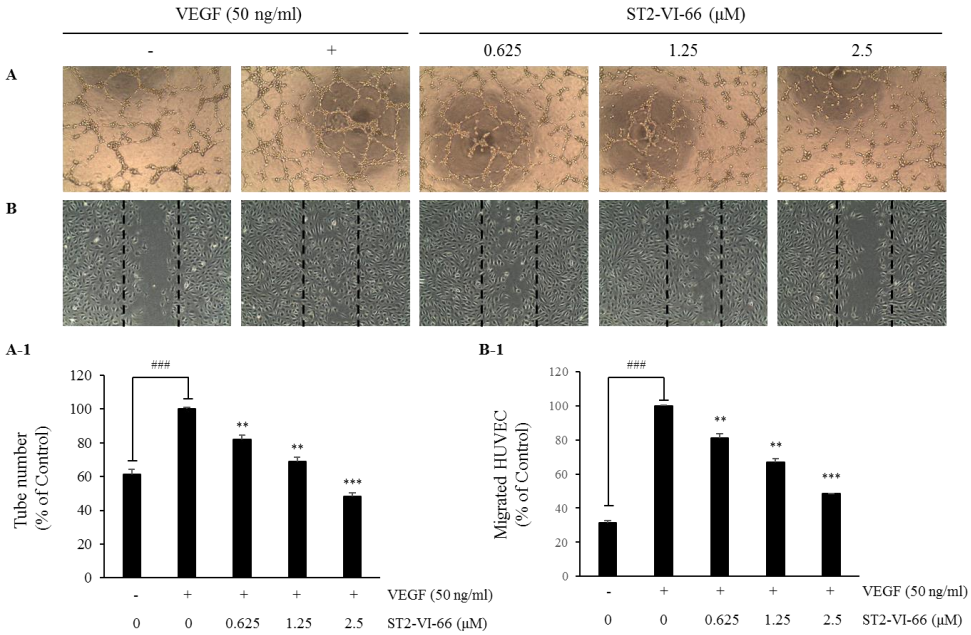

VEGF (50 ng/ml)
ST2-VI-66 (μM)
-
+
0.625
1.25
2.5
A
B
A-1
B-1
Tube number (% of Control)
Migrated HUVEC (% of Control)
###
**
***
VEGF (50 ng/ml)
ST2-VI-66 (μM)
0
0.625
1.25
2.5

Figure 19. The inhibitory effects ST2-VI-66 on cell migration and capillary-like tube formation

(A, A-1) In tube formation assay, HUVECs were seeded in Matrigel-coated 96-well plates. The cells were treated with VEGF (50 ng/ml) in the presence or absence of various concentrations of ST2-VI-66**.** After incubation for 4-8 h, the capillary structures were photographed and quantified. (B, B-1) In cell migration assay, HUVECs were grown to confluence in gelatin-coated 12-well plates and then scratched with a tip. The cells were treated with VEGF (50 ng/ml) in the presence or absence of various concentrations of ST2-VI-66 for 24 h. The migrated cells were counted and compared to the vehicle-treated control groups. The data were represented as the mean ± SD. ($^{\#\#\#}P < 0.001$; $^{**}P < 0.01$; $^{***}P < 0.001$)

13. Effect of ST2-VI-66 on the modulation of cell signaling pathways

To further elucidate the molecular mechanisms associated with the endothelial cell migration and tube formation, VEGF-induced biomarkers were analyzed with the treatment of ST2-VI-66 in HUVECs.

Phosphoinositide 3-kinase (PI3K)-AKT-endothelial nitric oxide synthase (eNOS) axis signaling pathway is considered one of the important molecular mechanisms in the VEGF-induced neoangiogenic processes in endothelial cells. VEGF is able to activate PI3K and then subsequently evoke the activation of the serine/threonine kinase AKT (Meadows et al., 2001). AKT also regulates the activation of endothelial NO synthase (eNOS), which is also associated with the regulation of vessel diameter (Claesson-Welsh and Welsh, 2013).

As shown in Figure 20, the treatment of VEGF (50 ng/ml) for 20 min to HUVECs led to the activation of PI3K (p-PI3K), AKT (p-AKT) and eNOS (p-eNOS), but the pre-treatment of ST2-VI-66 for 30 min significantly suppressed the activation processes in endothelial cells.

In addition, the activated VEGFR-2 (p-VEGFR-2) levels by VEGF were also suppressed by ST2-VI-66, and subsequently inhibited the activation of SRC (p-SRC)/FAK (p-FAK) in VEGF-stimulated HUVECs. It is well known that phosphorylation of VEGFR-2 (Tyr1175) affects to the activation of PI3K signaling and thus this event led to the increase of vascular permeability (Olsson et al., 2006).

In VEGF signaling pathway, SRC kinases are downstream of VEGFR2 and

regulate vascular permeability, angiogenesis (Eliceiri et al., 1999). The activation of FAK is also markedly involved in the endothelial cell migration, proliferation and adherence junction integrity (Brunton and Frame, 2008; Lechertier and Hodivala-Dilke, 2012). Therefore, the present data indicate that the inhibition of cell migration and vascular permeability by ST2-VI-66 is in part associated with the modulation of the signaling axis of VEGFR2-mediated PI3K/AKT/eNOS and SRC/FAK signaling pathway in HUVECs.

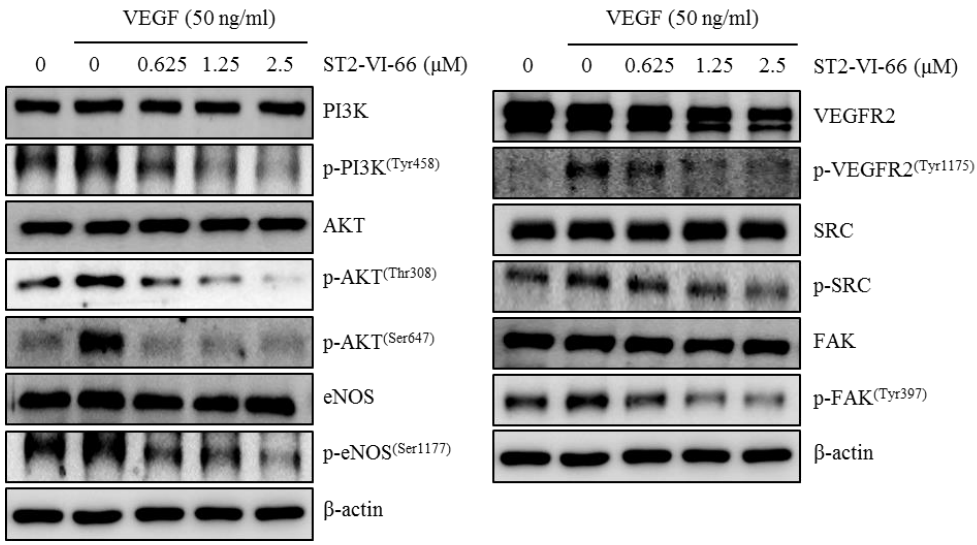


Figure 20. Effect of compound ST2-VI-66 on PI3K/AKT/eNOS and FAK signaling pathway in HUVECs

Cells were treated with various concentrations of ST2-VI-66 for 30 min and then stimulated with VEGF (50 ng/ml) for 20 min. The protein expressions were determined by western blotting as described in Experimental Section. β-Actin was used as a loading control.

14. Effect of ST2-VI-66 on hyaloid vessel in zebrafish

Human diabetes (Type 2) is able to induce chronic hyperglycaemia and diabetes retinopathy (DR). DR is also known to be correlated with the iris neovascularization. Since DR is associated with the angiogenic processes we tried to evaluate the effects of ST2-VI-66 on neovascular formation in DR-mimic animal model.

In general, DR drives to progressive vascular occlusions which lead to blindness with pericyte loss and vessel thickening. DR also increases vascular permeability which leads to macula edema (Bergers and Song, 2005).

To this end, zebrafish larvae treated with high glucose (HG) was employed for mimic to mammalian DR models (Jung et al., 2016). Transgenic zebrafish (*flk:EGFP*) embryos (3 dpf) were treated with 130 mM glucose for 3 days to induce DR-mimic hyaloid vessel in larvae eye lenses.

As shown in Figure 21, HG-treated zebrafish larvae significantly increased hyaloid vessel diameters in isolated eye lenses. However, the treatment of compound ST2-VI-66 effectively suppressed the increase of hyaloid vessel diameters induced by HG in a concentration-dependent manner. Especially, the treatment of 2.5 μM ST2-VI-66 markedly reduced the hyaloid vessel diameters at optic disc area.

These findings suggest that the reduction of HG-induced hyaloid vessel diameters by ST2-VI-66 might be also in part associated with the anti-angiogenic activity in *in vivo* animal model.

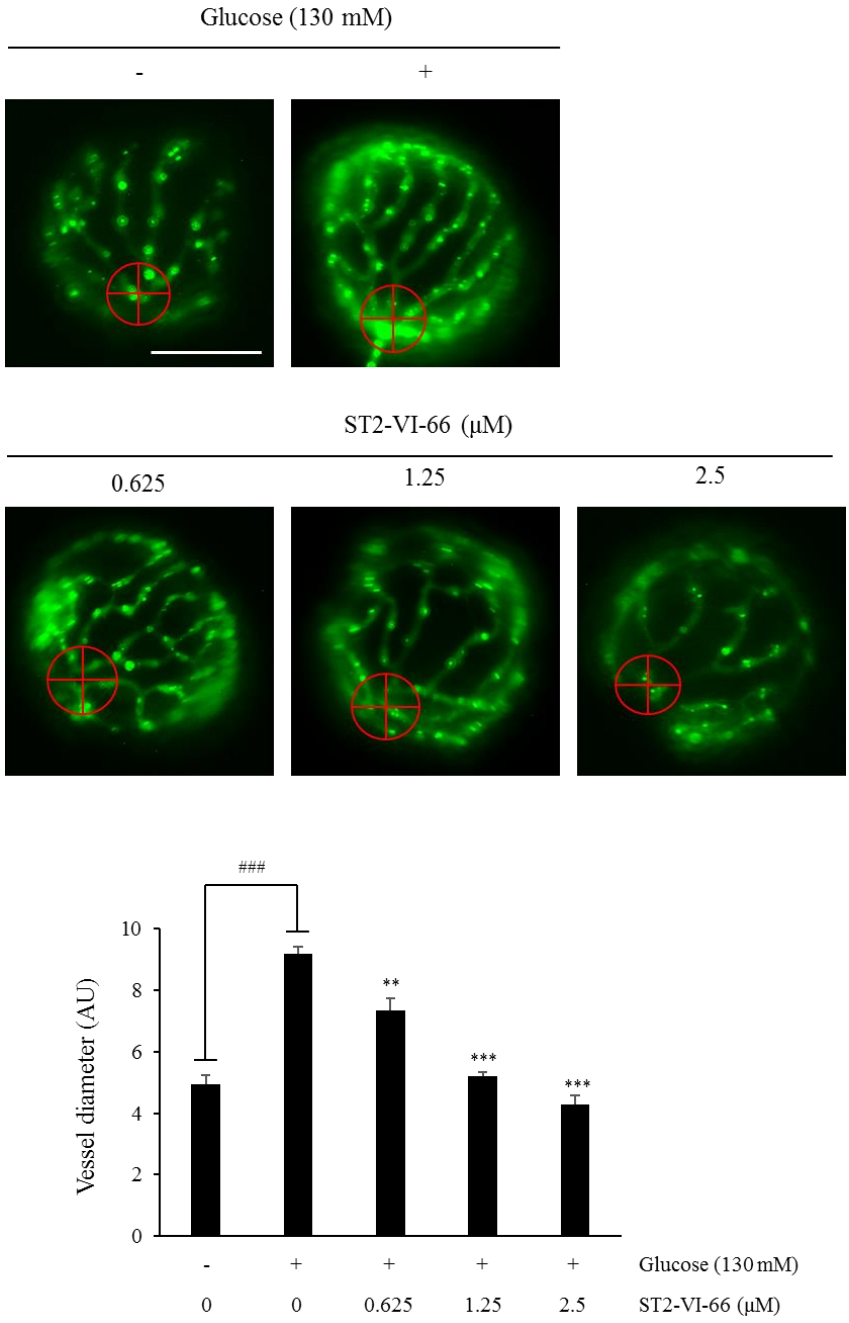

Glucose (130 mM)
-
+
ST2-VI-66 (μM)
0.625
1.25
2.5
###
**
***
***
Vessel diameter (AU)
10
8
6
4
2
0
-
+
+
+
+
Glucose (130 mM)
0
0
0.625
1.25
2.5
ST2-VI-66 (μM)

Figure 21. Effects of compound ST2-VI-66 on hyaloid vessels in HG-treated zebrafish larvae

Transgenic zebrafish (*flk:EGFP*) embryos were treated with high glucose (130 mM) in the presence or absence of ST2-VI-66 in 12-well plates. Optic disc branches from the isolated eye lenses of zebrafish larvae were visualized and photographed using fluorescence microscope. Hyaloid vessel diameters were measured at position near to the optic disc (red circle) using ImageJ software. Scale bar = 50 μm. The data are represented as the mean ± SD. $^{\#\#\#}P < 0.001$; $^{**}P < 0.01$; $^{***}P < 0.001$.

D. Discussion

Natural products provide valuable sources in the development of therapeutic agents. In study continuing efforts to identify new entities for anti-angiogenic agents from natural products, finding that wondoin, a new bis (dihydroxylstyryl) imidazole, has a potential inhibitory activity of angiogenesis (Jun et al., 2007; Shin et al., 2001). But, wondonin is difficult to extraction and synthesized. Hence, wondonin derivatives were synthesized base on the wondonin.

Angiogenesis is a complex multi-step process involving endothelial cell migration, proliferation, and capillary tube formation (Blood and Zetter, 1990). Endothelial cell function and angiogenesis are tightly regulated by growth factors, particularly by VEGF. VEGFR-2 has been reported to be the critical molecule responsible for maintaining endothelial normal (Bagri et al., 2010). Also, HIF-1α expression is associated with many common tumor-specific genetic alteration and this component is good anti-angiogenic target (Blagosklonny, 2001; Semenza, 2000b, 2003). Activation of HIF lead to expression of pro-angiogenic factors and these whole products are involved in angiogenesis, cell differentiation, migration, proliferation and vascular remodeling (Campochiaro, 2013; Semenza, 2000b). Especially, VEGF is highly stimulated by HIF-1α (Powis and Kirkpatrick, 2004).

Various VEGF inhibitors are on the market for treatment of cancer, these drugs are used anti-cancer drug and the potential to be used as therapeutic agents for obesity and diabetic retinopathy. However, the development of novel VEGF inhibitor wasn’t made for such diseases.

In order that treatment of other disease except cancer, need to development of the novel VEGF pathway inhibitor. To finding new compound that including the potent anti-angiogenesis along with a minimized cytotoxicity is important target.

In the process of angiogenesis, PI3K kinase is activated by various stimuli including VEGF in endothelial cells and regulate multiple critical steps by phosphorylating different downstream substrates, such as AKT and eNOS (Williams et al., 2000). The activation of the PI3K/AKT/eNOS pathway contributes to the VEGF-mediated stimulation of permeability of endothelial cells (Fulton et al., 1999). Also, VEGFR2 leads to the tyrosine phosphorylation of FAK that stimulated endothelial migration in response to VEGF (Garcia-Cardena and Folkman, 1998; Masson-Gadais et al., 2003; Rousseau et al., 2000b).

In the present study, therefore, effects of wondonin analogues evaluated on angiogenesis and elucidated the plausible mechanisms of actions in endothelial cells including HUVECs.

First, ST-XII-145 had most effective about anti-angiogenesis that among the wondonin analogues. So, ST-XII-145 selection and then investigated about anti-angiogenic effect. ST-XII-145 significantly inhibited migration and tube formation in VEGF-induced HUVECs and inhibited migration, tube formation, invasion in hypoxic condition. The anti-angiogenic activity of ST-XII-145 was also confirmed by the inhibition of capillary sprouting by endothelial cells in an *ex vivo* mouse aortic ring culture models. Next, ST-XII-145 evaluated their anti-angiogenic effect by zebrafish *in vivo* model. ST-XII-145 led to decreased length of SIV than vehicle group in wild type embryo and then similar effect was observed in transgenic

Tg(*flk:egfp*) embryo. Further study was designed to elucidate the involved mechanism of action for the ST-XII-145 mediated anti-angiogenic activity in endothelial cells. Also, ST-XII-145 effectively inhibited the capillary-like tube formation in cultured mES/EB-derived endothelial cells, which was well correlated with the suppression of the expression of the endothelial biomarker PECAM. The findings suggested that ST-XII-145 suppresses the VEGF-mediated PI3K/AKT/eNOS, FAK, p38 signaling pathway in HUVECs and these mechanism is presented similar pathway in hypoxic condition. The result was well correlated with the inhibition of VEGF-induced endothelial cell migration, invasion, and tubular formation by ST-XII-145.

The next structural modification against ST-XII-145 was made to synthesis another wondonin analogues. ST2-VI-66 has higher SI value and lower cytotoxicity than ST-XII-145.

ST2-VI-66 significantly inhibited migration and tube formation in VEGF-induced HUVECs. In addition, ST2-VI-66 effectively decreased the activation of VEGFR2, which subsequently led to the inhibition of PI3K/AKT/eNOS signaling. The present data suggest that ST2-VI-66 efficiently suppresses the activation of VEGF-stimulated FAK, thus resulting in the inhibited cell migration in endothelial cells. Further study was designed that ST2-VI-66 evaluated their anti-angiogenic effect in DR-mimic model used zebrafish.

In summary, ST2-VI-66 has a potential anti-angiogenic activity with the inhibition of migration and tube formation by endothelial cells. The underlying mechanisms

are regulation of the multiple steps of angiogenesis *via* modulation of PI3K/AKT/eNOS and FAK signaling pathways in endothelial cells.

Although anti-angiogenic activity of ST2-VI-66 with modulation of several biomarkers associated with cell migration and permeability was found, the precise mechanism of action of ST2-VI-66 is still unclear. To determine, anti-angiogenic effect of ST-VI-66, further detailed studies are needed to explore about VEGFR2 upstream signaling associated with HIF.

Therefore, these findings support the potential application of wondonin analogues for treatment of overactivation of angiogenesis including diabetes retinopathy.

References

Abdullah, S. E., and Perez-Soler, R. (2012). Mechanisms of resistance to vascular endothelial growth factor blockade. *Cancer* **118**, 3455-3467.

Abedi, H., and Zachary, I. (1997). Vascular endothelial growth factor stimulates tyrosine phosphorylation and recruitment to new focal adhesions of focal adhesion kinase and paxillin in endothelial cells. *J Biol Chem* **272**, 15442-15451.

Adair, T. H., and Montani, J.-P. (2010). Overview of angiogenesis.

Adamis, A. P., Miller, J. W., Bernal, M. T., D'Amico, D. J., Folkman, J., Yeo, T. K., and Yeo, K. T. (1994). Increased vascular endothelial growth factor levels in the vitreous of eyes with proliferative diabetic retinopathy. *Am J Ophthalmol* **118**, 445-450.

Agostinis, P., Vantieghem, A., Merlevede, W., and de Witte, P. A. (2002). Hypericin in cancer treatment: more light on the way. *Int J Biochem Cell Biol* **34**, 221-241.

Ahmed, A., Dunk, C., Ahmad, S., and Khaliq, A. (2000). Regulation of placental vascular endothelial growth factor (VEGF) and placenta growth factor

(PIGF) and soluble Flt-1 by oxygen--a review. *Placenta* **21 Suppl A**, S16-S24.

Archer, D. B. (1993). Doyne Lecture. Responses of retinal and choroidal vessels to ionising radiation. *Eye (Lond)* **7 (Pt 1)**, 1-13.

Ashton, N., Ward, B., and Serpell, G. (1954). Effect of oxygen on developing retinal vessels with particular reference to the problem of retrolental fibroplasia. *Br J Ophthalmol* **38**, 397-432.

Bagri, A., Kouros-Mehr, H., Leong, K. G., and Plowman, G. D. (2010). Use of anti-VEGF adjuvant therapy in cancer: challenges and rationale. *Trends Mol Med* **16**, 122-132.

Baker, M., Robinson, S. D., Lechertier, T., Barber, P. R., Tavora, B., D'Amico, G., Jones, D. T., Vojnovic, B., and Hodivala-Dilke, K. (2012). Use of the mouse aortic ring assay to study angiogenesis. *Nature protocols* **7**, 89-104.

Batra, P., and Sharma, A. K. (2013). Anti-cancer potential of flavonoids: recent trends and future perspectives. *3 Biotech* **3**, 439-459.

Bergers, G., and Song, S. (2005). The role of pericytes in blood-vessel formation and maintenance. *Neuro Oncol* **7**, 452-464.

Blagosklonny, M. V. (2001). Hypoxia-inducible factor: Achilles' heel of antiangiogenic cancer therapy (review). *Int J Oncol* **19**, 257-262.

Blood, C. H., and Zetter, B. R. (1990). Tumor interactions with the vasculature: angiogenesis and tumor metastasis. *Biochim Biophys Acta* **1032**, 89-118.

Brunton, V. G., and Frame, M. C. (2008). Src and focal adhesion kinase as therapeutic targets in cancer. *Curr Opin Pharmacol* **8**, 427-432.

Bunn, H. F., and Poyton, R. O. (1996). Oxygen sensing and molecular adaptation to hypoxia. *Physiol Rev* **76**, 839-885.

Campochiaro, P. A. (2013). Ocular neovascularization. *J Mol Med (Berl)* **91**, 311-321.

Carmeliet, P., and Jain, R. K. (2000). Angiogenesis in cancer and other diseases. *Nature* **407**, 249-257.

Carmeliet, P., and Jain, R. K. (2011). Molecular mechanisms and clinical applications of angiogenesis. *Nature* **473**, 298-307.

Carvalho, A., Costa, P., Vieira, G., Jamacaru, F., Moraes, M., Cavalcanti, B., and

Pessoa, C. (2011). Natural products used as candidates for angiogenesis inhibitors in cancer therapy. *Trends Org. Chem.* **15**, 79-93.

Chung, A. S., Lee, J., and Ferrara, N. (2010). Targeting the tumour vasculature: insights from physiological angiogenesis. *Nat Rev Cancer* **10**, 505-514.

Claesson-Welsh, L., and Welsh, M. (2013). VEGFA and tumour angiogenesis. *J Intern Med* **273**, 114-127.

Clauss, M., Sunderkotter, C., Sveinbjornsson, B., Hippenstiel, S., Willuweit, A., Marino, M., Haas, E., Seljelid, R., Scheurich, P., Suttorp, N., Grell, M., and Risau, W. (2001). A permissive role for tumor necrosis factor in vascular endothelial growth factor-induced vascular permeability. *Blood* **97**, 1321-1329.

Conway, E. M., Collen, D., and Carmeliet, P. (2001). Molecular mechanisms of blood vessel growth. *Cardiovasc Res* **49**, 507-521.

Cragg, G. M., and Newman, D. J. (2013). Natural products: a continuing source of novel drug leads. *Biochim Biophys Acta* **1830**, 3670-3695.

Curtis, T. M., Gardiner, T. A., and Stitt, A. W. (2009). Microvascular lesions of diabetic retinopathy: clues towards understanding pathogenesis? *Eye (Lond)*

23, 1496-1508.

Dayanir, V., Meyer, R. D., Lashkari, K., and Rahimi, N. (2001). Identification of tyrosine residues in vascular endothelial growth factor receptor-2/FLK-1 involved in activation of phosphatidylinositol 3-kinase and cell proliferation. *J Biol Chem* **276**, 17686-17692.

de Vogel, S., Dindore, V., van Engeland, M., Goldbohm, R. A., van den Brandt, P. A., and Weijenberg, M. P. (2008). Dietary folate, methionine, riboflavin, and vitamin B-6 and risk of sporadic colorectal cancer. *J Nutr* **138**, 2372-2378.

Dudkowska, M., and Kucharewicz, K. (2013). Natural compounds--modulators of senescence and cell death. *Postepy biochemii* **60**, 207-220.

Eliceiri, B. P., Paul, R., Schwartzberg, P. L., Hood, J. D., Leng, J., and Cheresh, D. A. (1999). Selective requirement for Src kinases during VEGF-induced angiogenesis and vascular permeability. *Mol Cell* **4**, 915-924.

Ferrara, N., and Bunting, S. (1996). Vascular endothelial growth factor, a specific regulator of angiogenesis. *Curr Opin Nephrol Hypertens* **5**, 35-44.

Folkman, J. (1995). Angiogenesis in cancer, vascular, rheumatoid and other disease. *Nat Med* **1**, 27-31.

Folkman, J., and Klagsbrun, M. (1987). Angiogenic factors. *Science* **235**, 442-447.

Fresco, P., Borges, F., Diniz, C., and Marques, M. P. (2006). New insights on the anticancer properties of dietary polyphenols. *Med Res Rev* **26**, 747-766.

Fujio, Y., and Walsh, K. (1999). Akt mediates cytoprotection of endothelial cells by vascular endothelial growth factor in an anchorage-dependent manner. *J Biol Chem* **274**, 16349-16354.

Fulton, D., Gratton, J. P., McCabe, T. J., Fontana, J., Fujio, Y., Walsh, K., Franke, T. F., Papapetropoulos, A., and Sessa, W. C. (1999). Regulation of endothelium-derived nitric oxide production by the protein kinase Akt. *Nature* **399**, 597-601.

Garcia-Cardena, G., and Folkman, J. (1998). Is there a role for nitric oxide in tumor angiogenesis? *J Natl Cancer Inst* **90**, 560-561.

Giordano, F. J., and Johnson, R. S. (2001). Angiogenesis: the role of the microenvironment in flipping the switch. *Curr Opin Genet Dev* **11**, 35-40.

Gore, A. V., Monzo, K., Cha, Y. R., Pan, W., and Weinstein, B. M. (2012). Vascular development in the zebrafish. *Cold Spring Harb Perspect Med* **2**, a006684.

Grossniklaus, H. E., Kang, S. J., and Berglin, L. (2010). Animal models of choroidal and retinal neovascularization. *Prog Retin Eye Res* **29**, 500-519.

Harris, A. L. (2000). von Hippel-Lindau syndrome: target for anti-vascular endothelial growth factor (VEGF) receptor therapy. *Oncologist* **5 Suppl 1**, 32-36.

Hirota, K., and Semenza, G. L. (2006). Regulation of angiogenesis by hypoxia-inducible factor 1. *Crit Rev Oncol Hematol* **59**, 15-26.

Hsin, C.-H., Wu, B.-C., Chuang, C.-Y., Yang, S.-F., Hsieh, Y.-H., Ho, H.-Y., Lin, H.-P., Chen, M.-K., and Lin, C.-W. (2013). Selaginella tamariscina extract suppresses TPA-induced invasion and metastasis through inhibition of MMP-9 in human nasopharyngeal carcinoma HONE-1 cells. *BMC complement Altern Med* **13**, 234.

Huang, L. E., Arany, Z., Livingston, D. M., and Bunn, H. F. (1996). Activation of hypoxia-inducible transcription factor depends primarily upon redox-sensitive stabilization of its alpha subunit. *J Biol Chem* **271**, 32253-32259.

Josko, J., Gwozdz, B., Jedrzejowska-Szypulka, H., and Hendryk, S. (2000). Vascular endothelial growth factor (VEGF) and its effect on angiogenesis. *Med Sci Monit* **6**, 1047-1052.

Jun, H. O., Kim, Y., Kwon, Y. W., Hong, S. S., Kim, K. W., Shin, J., and Kim, T. Y. (2007). Wondonin, a novel compound, inhibits hypoxia-induced angiogenesis through hypoxia-inducible factor 1 alpha. *FEBS Lett* **581**, 4977-4982.

Jung, S. H., Kim, Y. S., Lee, Y. R., and Kim, J. S. (2016). High glucose-induced changes in hyaloid-retinal vessels during early ocular development of zebrafish: a short-term animal model of diabetic retinopathy. *Br J Pharmacol* **173**, 15-26.

Kallio, P. J., Pongratz, I., Gradin, K., McGuire, J., and Poellinger, L. (1997). Activation of hypoxia-inducible factor 1alpha: posttranscriptional regulation and conformational change by recruitment of the Arnt transcription factor. *Proc Natl Acad Sci U S A* **94**, 5667-5672.

Kim, E.-C., Kim, S. H., Piao, S.-J., Kim, T.-J., Bae, K., Kim, H. S., Hong, S.-S., Lee, B. I., and Nam, M. (2015). Antiangiogenic activity of Acer tegmentosum maxim water extract in vitro and in vivo. *J Korean Med Sci* **30**, 979-987.

King, A., Balaji, S., Keswani, S. G., and Crombleholme, T. M. (2014). The role of stem cells in wound angiogenesis. *Adv Wound Care* **3**, 614-625.

Kinja, K., Rohit, S., Mandloi, A., Sharma, I., and Savita, S. (2011). A Review on "Anti-angiogenic Therapy - Past, Present and Future". *Rec Res Sci Tech* **3**, 8-15.

Kruger, E. A., Duray, P. H., Tsokos, M. G., Venzon, D. J., Libutti, S. K., Dixon, S. C., Rudek, M. A., Pluda, J., Allegra, C., and Figg, W. D. (2000). Endostatin inhibits microvessel formation in the ex vivo rat aortic ring angiogenesis assay. *Biochem Biophys Res Commun* **268**, 183-191.

Lau, S. T., Lin, Z. X., Zhao, M., and Leung, P. S. (2008). Brucea javanica fruit induces cytotoxicity and apoptosis in pancreatic adenocarcinoma cell lines. *Phytother Res* **22**, 477-486.

Le Boeuf, F., Houle, F., and Huot, J. (2004). Regulation of vascular endothelial growth factor receptor 2-mediated phosphorylation of focal adhesion kinase by heat shock protein 90 and Src kinase activities. *J Biol Chem* **279**, 39175-39185.

Lechertier, T., and Hodivala-Dilke, K. (2012). Focal adhesion kinase and tumour angiogenesis. *J Pathol* **226**, 404-412.

Lee, R. T., Hlubocky, F. J., Hu, J. J., Stafford, R. S., and Daugherty, C. K. (2008a). An international pilot study of oncology physicians' opinions and practices

on Complementary and Alternative Medicine (CAM). *Integr Cancer Ther* **7**, 70-75.

Lee, S. M., Kwon, J. I., Choi, Y. H., Eom, H. S., and Chi, G. Y. (2008b). Induction of G2/M arrest and apoptosis by water extract of Strychni Semen in human gastric carcinoma AGS cells. *Phytother Res* **22**, 752-758.

Lenzi, P., Bocci, G., and Natale, G. (2016). John Hunter and the origin of the term "angiogenesis". *Angiogenesis* **19**, 255-256.

Livak, K., and Schmittgen, T. (2001). Analysis of relative gene expression data using real-time quantitative PCR and the 2− ΔΔCT method. Methods **25** 402–408.

Lohela, M., Bry, M., Tammela, T., and Alitalo, K. (2009). VEGFs and receptors involved in angiogenesis versus lymphangiogenesis. *Curr Opin Cell Biol* **21**, 154-165.

Majumdar, S., and Srirangam, R. (2010). Potential of the bioflavonoids in the prevention/treatment of ocular disorders. *J Pharm Pharmacol* **62**, 951-965.

Manach, C., Scalbert, A., Morand, C., Remesy, C., and Jimenez, L. (2004). Polyphenols: food sources and bioavailability. *Am J Clin Nutr* **79**, 727-747.

Masson-Gadais, B., Houle, F., Laferrière, J., and Huot, J. (2003). Integrin αvβ3 requirement for VEGFR2-mediated activation of SAPK2/p38 and for Hsp90-dependent phosphorylation of focal adhesion kinase in endothelial cells activated by VEGF. *Cell Stress Chaperones* **8**, 37-52.

Matsumoto, T., Bohman, S., Dixelius, J., Berge, T., Dimberg, A., Magnusson, P., Wang, L., Wikner, C., Qi, J. H., Wernstedt, C., Wu, J., Bruheim, S., Mugishima, H., Mukhopadhyay, D., Spurkland, A., and Claesson-Welsh, L. (2005). VEGF receptor-2 Y951 signaling and a role for the adapter molecule TSAd in tumor angiogenesis. *EMBO J* **24**, 2342-2353.

McIntosh, R. L., Rogers, S. L., Lim, L., Cheung, N., Wang, J. J., Mitchell, P., Kowalski, J. W., Nguyen, H. P., and Wong, T. Y. (2010). Natural history of central retinal vein occlusion: an evidence-based systematic review. *Ophthalmology* **117**, 1113-1123 e15.

McMullen, M. E., Bryant, P. W., Glembotski, C. C., Vincent, P. A., and Pumiglia, K. M. (2005). Activation of p38 has opposing effects on the proliferation and migration of endothelial cells. *J Biol Chem* **280**, 20995-21003.

Meadows, K. N., Bryant, P., and Pumiglia, K. (2001). Vascular endothelial growth factor induction of the angiogenic phenotype requires Ras activation. *J Biol Chem* **276**, 49289-49298.

Mehta, H. J., Patel, V., and Sadikot, R. T. (2014). Curcumin and lung cancer--a review. *Target Oncol* **9**, 295-310.

Michiels, C., Arnould, T., and Remacle, J. (2000). Endothelial cell responses to hypoxia: initiation of a cascade of cellular interactions. *Biochim Biophys Acta* **1497**, 1-10.

Miller, J. W. (1997). Vascular endothelial growth factor and ocular neovascularization. *Am J Pathol* **151**, 13-23.

Miller, J. W., Le Couter, J., Strauss, E. C., and Ferrara, N. (2013). Vascular endothelial growth factor a in intraocular vascular disease. *Ophthalmology* **120**, 106-114.

Najda, A., Dyduch-Sieminska, M., Dyduch, J., and Gantner, M. (2014). Comparative analysis of secondary metabolites contents in Fragaria vesca L. fruits. *Ann Agric Environ Med* **21**, 339-343.

Neufeld, G., Cohen, T., Gengrinovitch, S., and Poltorak, Z. (1999). Vascular endothelial growth factor (VEGF) and its receptors. *FASEB J* **13**, 9-22.

Olsson, A. K., Dimberg, A., Kreuger, J., and Claesson-Welsh, L. (2006). VEGF

receptor signalling - in control of vascular function. *Nat Rev Mol Cell Biol* **7**, 359-371.

Pages, G., Milanini, J., Richard, D. E., Berra, E., Gothie, E., Vinals, F., and Pouyssegur, J. (2000). Signaling angiogenesis via p42/p44 MAP kinase cascade. *Ann N Y Acad Sci* **902**, 187-200.

Powis, G., and Kirkpatrick, L. (2004). Hypoxia inducible factor-1alpha as a cancer drug target. *Mol Cancer Ther* **3**, 647-654.

Risau, W. (1997). Mechanisms of angiogenesis. *Nature* **386**, 671-674.

Rousseau, S., Houle, F., and Huot, J. (2000a). Integrating the VEGF signals leading to actin-based motility in vascular endothelial cells. *Trends Cardiovasc Med* **10**, 321-327.

Rousseau, S., Houle, F., Kotanides, H., Witte, L., Waltenberger, J., Landry, J., and Huot, J. (2000b). Vascular endothelial growth factor (VEGF)-driven actin-based motility is mediated by VEGFR2 and requires concerted activation of stress-activated protein kinase 2 (SAPK2/p38) and geldanamycin-sensitive phosphorylation of focal adhesion kinase. *J Biol Chem* **275**, 10661-10672.

Rousseau, S., Houle, F., Landry, J., and Huot, J. (1997). p38 MAP kinase activation

by vascular endothelial growth factor mediates actin reorganization and cell migration in human endothelial cells. *Oncogene* **15**, 2169-2177.

Sagar, S. M., Yance, D., and Wong, R. K. (2006a). Natural health products that inhibit angiogenesis: a potential source for investigational new agents to treat cancer-Part 1. *Curr Oncol* **13**, 14-26.

Sagar, S. M., Yance, D., and Wong, R. K. (2006b). Natural health products that inhibit angiogenesis: a potential source for investigational new agents to treat cancer-Part 2. *Curr Oncol* **13**, 99-107.

Semenza, G. L. (2000a). HIF-1: mediator of physiological and pathophysiological responses to hypoxia. *J Appl Physiol (1985)* **88**, 1474-1480.

Semenza, G. L. (2000b). Hypoxia, clonal selection, and the role of HIF-1 in tumor progression. *Crit Rev Biochem Mol Biol* **35**, 71-103.

Semenza, G. L. (2003). Targeting HIF-1 for cancer therapy. *Nat Rev Cancer* **3**, 721-732.

Shahneh, F. Z., Baradaran, B., Zamani, F., and Aghebati-Maleki, L. (2013). Tumor angiogenesis and anti-angiogenic therapies. *Hum Antibodies* **22**, 15-19.

Shima, D. T., Gougos, A., Miller, J. W., Tolentino, M., Robinson, G., Adamis, A. P., and D'Amore, P. A. (1996). Cloning and mRNA expression of vascular endothelial growth factor in ischemic retinas of Macaca fascicularis. *Invest Ophthalmol Vis Sci* **37**, 1334-1340.

Shin, J., Rho, J.-R., Seo, Y., Lee, H.-S., Cho, K. W., Kwon, H. J., and Sim, C. J. (2001). Wondonins A and B, new bis (dihydroxystyryl) imidazoles from a two-sponge association. *Tetrahedron Lett* **42**, 1965-1968.

Shu, X., Wu, W., Mosteller, R. D., and Broek, D. (2002). Sphingosine kinase mediates vascular endothelial growth factor-induced activation of ras and mitogen-activated protein kinases. *Mol Cell Biol* **22**, 7758-7768.

Shukla, S., and Gupta, S. (2010). Apigenin: a promising molecule for cancer prevention. *Pharm Res* **27**, 962-978.

Tai, J., Cheung, S., Wu, M., and Hasman, D. (2012). Antiproliferation effect of Rosemary (Rosmarinus officinalis) on human ovarian cancer cells in vitro. *Phytomedicine* **19**, 436-443.

Takahashi, T., Yamaguchi, S., Chida, K., and Shibuya, M. (2001). A single autophosphorylation site on KDR/Flk-1 is essential for VEGF-A-dependent activation of PLC-gamma and DNA synthesis in vascular endothelial cells.

EMBO J **20**, 2768-2778.

Tang, N., Wang, L., Esko, J., Giordano, F. J., Huang, Y., Gerber, H. P., Ferrara, N., and Johnson, R. S. (2004). Loss of HIF-1alpha in endothelial cells disrupts a hypoxia-driven VEGF autocrine loop necessary for tumorigenesis. *Cancer Cell* **6**, 485-495.

Taraphdar, A. K., Roy, M., and Bhattacharya, R. (2001). Natural products as inducers of apoptosis: Implication for cancer therapy and prevention. *Curr Sci* **80**, 1387-1396.

Tolentino, M. J., Miller, J. W., Gragoudas, E. S., Chatzistefanou, K., Ferrara, N., and Adamis, A. P. (1996). Vascular endothelial growth factor is sufficient to produce iris neovascularization and neovascular glaucoma in a nonhuman primate. *Arch Ophthalmol* **114**, 964-970.

Tozer, G. M., Kanthou, C., and Baguley, B. C. (2005). Disrupting tumour blood vessels. *Nat Rev Cancer* **5**, 423-435.

Wang, G. L., Jiang, B. H., Rue, E. A., and Semenza, G. L. (1995). Hypoxia-inducible factor 1 is a basic-helix-loop-helix-PAS heterodimer regulated by cellular O2 tension. *Proc Natl Acad Sci U S A* **92**, 5510-5514.

Wenger, R. H. (2002). Cellular adaptation to hypoxia: O2-sensing protein hydroxylases, hypoxia-inducible transcription factors, and O2-regulated gene expression. *FASEB J* **16**, 1151-1162.

Weston, C. R., and Davis, R. J. (2002). The JNK signal transduction pathway. *Curr Opin Genet Dev* **12**, 14-21.

Williams, M. R., Arthur, J. S., Balendran, A., van der Kaay, J., Poli, V., Cohen, P., and Alessi, D. R. (2000). The role of 3-phosphoinositide-dependent protein kinase 1 in activating AGC kinases defined in embryonic stem cells. *Curr Biol* **10**, 439-448.

Yi, J.-M., Park, J.-S., Oh, S.-M., Lee, J., Kim, J., Oh, D.-S., Bang, O.-S., and Kim, N. S. (2012). Ethanol extract of Gleditsia sinensis thorn suppresses angiogenesis in vitro and in vivo. *BMC Complement Altern Med* **12**, 243.

Zeng, H., Sanyal, S., and Mukhopadhyay, D. (2001). Tyrosine residues 951 and 1059 of vascular endothelial growth factor receptor-2 (KDR) are essential for vascular permeability factor/vascular endothelial growth factor-induced endothelium migration and proliferation, respectively. *J Biol Chem* **276**, 32714-32719.

Zhang, J., Jiang, X., Jiang, Y., Guo, M., Zhang, S., Li, J., He, J., Liu, J., Wang, J.,

and Ouyang, L. (2016). Recent advances in the development of dual VEGFR and c-Met small molecule inhibitors as anticancer drugs. *Eur J Med Chem* **108**, 495-504.

Zhang, S. X., and Ma, J. X. (2007). Ocular neovascularization: Implication of endogenous angiogenic inhibitors and potential therapy. *Prog Retin Eye Res* **26**, 1-37.

국문초록

Wondonin 유도체의 혈관신생 억제 작용기전 연구

오 제 도
약학과 천연물과학전공
서울대학교 약학대학 대학원

성인 또는 태아의 발달에 신생혈관형성은 필수적인 과정이다. 하지만, 과도하거나 불충분한 혈관형성은 cancer, age-related macular degeneration (AMD), diabetic retinopathy, cardio vascular disease 등 다양한 질병의 원인이 되고 있다. 이와 같은 비정상적 혈관형성으로 인한 질병치료를 위해 많은 연구가 진행되고 있다. 다음의 연구에서는 해양 천연물에서 유래된 wondonin을 기반으로 하여 합성한 유도체를 이용해 우수한 혈관형성억제효능에 대해 연구를 진행하였다.

본 연구는 wondonin 유도체의 혈관신생억제 효능과 작용기전을 밝히고자 하였다. 이에 세포에 독성이 없으며, 효과적으로 angiogenesis를 억제하는 물질을 찾고자 하였다. 독성검사와 혈관형성의 지표가 되는 tube formation assay를 통해 원 물질인 wondonin과의 활성 및 선별지수를 종합하여 물질 선별하였다.

선별한 유도체 중 ST-XII-145이 VEGF유도 및 hypoxia조건에서 PI3K/AKT/eNOS, FAK 및 p38의 단백질 발현을 효과적으로 억제함을 확인하였다. 이와 같은 *in-vitro*상의 결과를 바탕으로 mouse aorta ring assay (*ex-vivo*), zebrafish angiogenesis assay (*in-vivo*)를 통해 실제적으로 혈관신생억제능력이 있음을 관찰할 수 있었다. 또한 발생과정에서의 혈관형성억제 효능을 보기 위해 mouse 배아줄기세포를 내피세포로 분화시켜 Immunocychemistry방법을 이용하여 활성을 확인하였다. 이후 ST-XII-145 물질을 기반으로 하여 조금 더 나은 물질을 찾고자 시도하였다. 물질의 독성을 낮추고, 활성을 높이기 위해 구조변화를 통해 다양한 유도체들을 합성하였다. 합성된 유도체 중 ST2-VI-66이 높은 SI value 및 활성이 뛰어나 선택하였다.

ST2-VI-66의 혈관억제효능을 탐색한 결과 PI3K-AKT-eNOS 및 FAK 신호경로를 억제하는 것을 확인하였고, 혈관신생억제를 통한 질병치료의 응용성을 확보하기위해 당뇨성망막병증 모델을 zebrafish에 적용하였다. 그 결과 고혈당에 증가된 혈관 diameter가 효과적으로 물질에 의해 감소함을 확인 할 수 있었다.

이러한 결과들을 바탕으로 wondonin 유도체가 VEGF신호전달체계를 저해하여 과도한 신생혈관 생성으로 발생하는 질병들을 극복할 수 있는 후보 선도물질로서의 가능성을 제시한다.

주요어 : 혈관 신생, VEGF signaling, 원도닌 유도체, Zebrafish, mouse 배아줄기세포, 혈관내피세포

학번: 2012-21599