MECHANISTIC AND THERAPEUTIC EVALUATION OF A NOVEL ANTIANTIOGENIC SMALL MOLECULE

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DEDICATION

This dissertation is dedicated to my wonderful mother, Amal Ahmed, whose unconditional love, irreplaceable emotional support and exceptionally strong faith made me the person I am today and made this journey possible.

It is also dedicated to my amazing family, my father, Sulaiman Abdelgaber, my siblings, Dalia, Ghada, and Mohamed, and my nephew, Selim, for being there for me along the way, and being blessed with having them in my life will always make me grateful.
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reagents used in this study. I would like to thank Keith Condon, Indiana University School of Medicine Histology Core for his help sectioning the eye samples and carrying out H&E staining. Thanks to the lipidomics core facility at Wayne State University for performing and analyzing the lipid profiling experiment.

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Choroidal neovascularization (CNV) is the vision-threatening characteristic of wet age-related macular degeneration (AMD), a major cause of blindness affecting almost 2 million elderly Americans. The current approved treatments target the dominant angiogenic mediator, vascular endothelial growth factor (VEGF). However, repeated injections of anti-VEGF drugs can cause ocular and systemic side effects, and about 30% of wet AMD patients are non-responsive. There is thus an unmet need to develop VEGF-independent antiangiogenic molecules to complement or combine with existing medications.

I studied SH-11037, a novel homoisoflavonoid with potent and selective antiangiogenic activity against human retinal endothelial cells. Intravitreal SH-11037 dose-dependently suppressed angiogenesis in the laser-induced CNV (L-CNV) mouse model. These effects were prominent as early as 7 days post-laser treatment as measured by a novel ellipsoid quantification method of optical coherence tomography images in vivo. A supratherapeutic dose of 100 µM SH-11037 was not associated with signs of murine ocular toxicity, and did not interfere with pre-existing retinal vasculature or retinal function. SH-11037 synergized with anti-VEGF therapy in vitro and in vivo, suggesting a VEGF-independent mechanism. By photoaffinity pulldown, I identified soluble epoxide
hydrolase (sEH) as an SH-11037-binding target. sEH is a key enzyme in ω-3 and ω-6 fatty acid metabolism. sEH levels were dramatically upregulated in retinal sections from L-CNV mice and a specific sEH inhibitor, t-AUCB, significantly suppressed L-CNV lesion volume. Additionally, SH-11037 inhibited sEH enzymatic activity in vitro and in vivo in L-CNV mice. Given the role of sEH in the metabolism of docosahexaenoic acids (DHA), inhibition of sEH using small molecules like SH-11037 would enhance ocular DHA levels, with beneficial antiangiogenic and anti-inflammatory effects. SH-11037 is thus a novel sEH inhibitor, which could make it an alternative or additive therapy to existing anti-VEGF drugs for treatment of neovascular diseases in the eye and other tissues.

Timothy W. Corson, Ph.D., Chair
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<tbody>
<tr>
<td>3D</td>
<td>Three-dimensional</td>
</tr>
<tr>
<td>AMD</td>
<td>Age-related macular degeneration</td>
</tr>
<tr>
<td>Apo E</td>
<td>Apolipoprotein E</td>
</tr>
<tr>
<td>AR</td>
<td>Affinity reagent</td>
</tr>
<tr>
<td>ARMS2</td>
<td>Age-related maculopathy susceptibility 2 gene</td>
</tr>
<tr>
<td>BAECs</td>
<td>Bovine aortic endothelial cells</td>
</tr>
<tr>
<td>CDK</td>
<td>Cyclin-dependent kinase</td>
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<tr>
<td>CFH</td>
<td>Complement factor H</td>
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<tr>
<td>CNV</td>
<td>Choroidal neovascularization</td>
</tr>
<tr>
<td>Combo</td>
<td>Combination</td>
</tr>
<tr>
<td>COX2</td>
<td>Cyclooxygenase 2</td>
</tr>
<tr>
<td>DHA</td>
<td>Docosahexaenoic acid</td>
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<td>DHDP</td>
<td>Dihydroxydocosapentaenoic acid</td>
</tr>
<tr>
<td>DHET</td>
<td>Dihydroxyeicosatrienoic acid</td>
</tr>
<tr>
<td>DMSO</td>
<td>Dimethyl sulfoxide</td>
</tr>
<tr>
<td>EBM</td>
<td>Endothelial basal medium</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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<tr>
<td>--------------</td>
<td>-----------</td>
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<tr>
<td>ECM</td>
<td>Extracellular matrix</td>
</tr>
<tr>
<td>EDP</td>
<td>Epoxydocosapentaenoic acid</td>
</tr>
<tr>
<td>EdU</td>
<td>5-ethynyl-2'-deoxyuridine</td>
</tr>
<tr>
<td>EET</td>
<td>Epoxeyicosatrienoic acid</td>
</tr>
<tr>
<td>EGM</td>
<td>Endothelial growth medium</td>
</tr>
<tr>
<td>EPA</td>
<td>Eicosapentaenoic acid</td>
</tr>
<tr>
<td>ERG</td>
<td>Electoretinogram</td>
</tr>
<tr>
<td>FA</td>
<td>Fluorescein angiography</td>
</tr>
<tr>
<td>FBS</td>
<td>Fetal bovine serum</td>
</tr>
<tr>
<td>FGF</td>
<td>Fibroblast growth factor</td>
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<tr>
<td>GCL</td>
<td>Ganglion cell layer</td>
</tr>
<tr>
<td>GFAP</td>
<td>Glial fibrillary acidic protein</td>
</tr>
<tr>
<td>H&amp;E</td>
<td>Hematoxylin and eosin</td>
</tr>
<tr>
<td>hEGF</td>
<td>human epidermal growth factor</td>
</tr>
<tr>
<td>hFGF</td>
<td>human fibroblast growth factor</td>
</tr>
<tr>
<td>HIF1α</td>
<td>Hypoxia-inducible factor 1α</td>
</tr>
<tr>
<td>HRECs</td>
<td>Human retinal microvascular endothelial cells</td>
</tr>
</tbody>
</table>
HSA: Highest single agent

HUVECs: Human umbilical vein endothelial cells

IF: Intermediate filament

IL: Interleukin

INL: Inner nuclear layer

IPL: Inner plexiform layer

IS: Inner segment of photoreceptors

L-CNV: Laser-induced choroidal neovascularization

MAPK: Mitogen activated protein kinase

MCP-1: Monocyte chemotactic protein-1

MMP: Matrix metalloproteases

NC: Negative control

NF-κB: Nuclear factor-kappa B

NO: Nitric oxide

OCT: Optical coherence tomography

OIR: Oxygen-induced retinopathy

ONH: Optic nerve head
ONL: Outer nuclear layer
OPL: Outer plexiform layer
OS: Outer segment of photoreceptors
PAGE: Polyacrylamide gel electrophoresis
PBS: Phosphate buffered saline
PDR: Proliferative diabetic retinopathy
PDT: Photodynamic therapy
PEDF: Pigment epithelium derived factor
PFA: Paraformaldehyde
PHD: Prolyl hydroxylase domain protein
PHOME: (3-phenyl-oxiranyl)-acetic acid cyano-(6-methoxy-naphthalen-2-yl)-methyl ester
PIGF: Placental growth factor
PPAR-γ: Peroxisome proliferator-activated receptor-γ
PUFA: Polyunsaturated fatty acid
PVDF: Polyvinylidene difluoride
R3-IGF-1: R3-insulin-like growth factor
ROP: Retinopathy of prematurity
RPE: Retinal pigment epithelium
SAR: Structure-activity relationship
SDS: Sodium dodecyl sulfate
sEH: Soluble epoxide hydrolase
SOD2: Superoxide dismutase 2
SP: Staurosporine
STZ: Streptozotocin
\( \text{t-AUCB}: \) trans-4-(4-[3-adamantan-1-yl-ureido]-cyclohexyloxy)-benzoic acid
TBS: Tris buffered saline
TBST: Tris buffered saline with Tween-20
TNF-\( \alpha \): Tumor necrosis factor-\( \alpha \)
UPP: Ubiquitin proteasome pathway
VEGF: Vascular endothelial growth factor
VEGFR: Vascular endothelial growth factor receptor
CHAPTER I. INTRODUCTION

(The majority of this chapter was previously published as the following review article: Sulaiman RS, Basavarajappa HD, and Corson TW. Natural product inhibitors of ocular angiogenesis. Experimental Eye Research, 2014, 129, 161-171 – reproduced with permission from Elsevier Limited)
A. Angiogenesis in Ocular Health and Disease

Angiogenesis is a highly regulated process that involves the formation of new blood vessels from existing ones (Carmeliet and Jain 2011). This process is clearly different from vasculogenesis, the de novo synthesis of blood vessels from endothelial progenitor cells. Angiogenesis does not only require endothelial cell migration and proliferation, but also vessel maturation, vessel remodeling, and degradation of extracellular matrix (ECM). Angiogenesis is the major mechanism of vascular growth during embryonic development and wound healing (Munoz-Chapuli et al. 2004). Under normal conditions, endothelial cells are quiescent without significant proliferation, due to a balance in the expression level and function of angiogenic factors such as vascular endothelial growth factor (VEGF) and angiostatic factors such as pigment epithelium derived factor (PEDF) (Folkman and Ingber 1992). The formation of new blood vessels in angiogenesis begins with the local destruction of pre-existing vessel walls followed by the activation of endothelial cell proliferation and migration. Stimulation of cell-cell adhesion facilitates the assembly of endothelial cells into tubular structures. Endothelial cells are then surrounded and stabilized by blood vessel walls consisting of a single layer of cells called pericytes, which suppress the proliferation of endothelial cells and release cell-survival factors such as VEGF (Karamysheva 2008, Carmeliet and Jain 2011).

Abnormal angiogenesis is implicated in various pathological conditions including tumor growth, metastasis, arthritis, and blinding eye diseases (Folkman
During rapid uncontrolled ocular angiogenesis, fragile and leaky vasculature is formed. This leads to hemorrhage and accumulation of fluids and protein exudates in ocular cavities, causing 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 (Zhang and Ma 2007).

The most dominant angiogenic factor in neovascularization is VEGF. Other growth factors such as fibroblast growth factor (FGF), and angiogenin mediate VEGF-independent mechanisms. Although the origins and etiologies of neovascular eye diseases are different, the mechanisms of neovascularization share some common pathways with other tissues as well. The balance between endogenous pro- and antiangiogenic factors tightly regulates homeostasis of ocular vasculature. When this balance is disturbed, formation of new blood vessels occurs that is implicated in neovascular eye diseases, which are a major cause of blindness throughout life. These diseases are characterized by the development of new blood vessels in the eye — either retinal or choroidal neovascularization — where blood vessels grow into the sensory retina, causing hemorrhage and severe vision impairment (de Jong 2006).

B. Retinal Neovascularization

The avascular characteristics of certain ocular compartments, including cornea, lens and the outer retina, is an important anatomical feature to satisfy
normal visual function. Therefore, in healthy adults, the ocular vasculature is mainly quiescent under the control of endogenous antiangiogenic factors such as PEDF and angiostatin (Qazi et al. 2009). The abnormal growth of new blood vessels in avascular areas of the retina, such as retinal neovascularization, interferes with their normal function in regulating light transmission. Numerous clinical and experimental studies have identified ischemia as the major cause of retinal neovascularization (Ashton et al. 1954). One of the most common forms of retinal neovascularization is proliferative diabetic retinopathy (PDR), a common cause of blindness in patients between 25 and 65 years old (Yau et al. 2012). Persistent hyperglycemia, high blood pressure and hypoxia in diabetic patients contribute to retinopathy and damage to retinal capillaries. This is followed by a proliferative stage, where new, abnormal blood vessels grow through the retina; this is sometimes exacerbated by the formation of fibrovascular scarring and retinal detachment (Grossniklaus et al. 2010). With an increasing diabetic population in the United States, approximately 700,000 Americans have diabetic retinopathy, with an annual incidence of 65,000 new cases (Zhang et al. 2010).

Retinopathy of prematurity (ROP) is another form of retinal neovascularization that is developmental in origin (Smith 2002). Retinal vascularization starts at approximately the 16th week of gestation and completes by full-term pregnancy (i.e. 40th week of gestation). Hence, premature babies have incomplete retinal vasculature and upon exposure to oxygen therapy (hyperoxia) during neonatal intensive care, this incomplete retinal vasculature
decays. This condition is more pronounced in premature babies with birth weight less than 1250 g (Sapieha et al. 2010). During this hyperoxia, the expression of hypoxia-driven angiogenic factors is down-regulated, resulting in a retardation of existing retinal blood vessel growth, which increases the metabolic requirement of the retina on return to normoxia, stimulating abnormal blood vessel formation. ROP severity ranges from mild with no visual defects to aggressive neovascularization causing blindness and retinal detachment that is responsible for 6 - 18% of total childhood blindness cases (Coats 2005). Vision loss in about 1300 children every year has been estimated in the United States alone, with many more cases worldwide, especially as survival of premature infants increases in developing countries (Javitt et al. 1993).

C. Choroidal Neovascularization and Wet Age-related Macular Degeneration

The choroid, a highly vascularized compartment of the eye responsible for delivering nutrients and oxygen to the photoreceptors, is also susceptible to neovascularization. In this case, aberrant vasculature can originate from the choriocapillaris and grow through a break in Bruch’s membrane to the sub-retinal space precipitating vision loss (Figure 1). This break may be secondary to trauma, a degenerative process, and/or tissue inflammation (Grossniklaus and Green 2004). Choroidal neovascularization (CNV) is most commonly seen in the wet (exudative) form of age-related macular degeneration (AMD), the leading cause of vision loss among people over 55 years old (Jager et al. 2008). Almost 2 million elderly Americans are affected by wet AMD, with about 200,000 new
cases diagnosed every year in the United States (Congdon et al. 2004, Fine et al. 2000b). Wet AMD has an estimated loss of productivity burden of $5.4$ billion annually in the United States (Brown et al. 2005). CNV can lead to swelling and damage of the macula, causing vision loss in the center of the retina.

AMD is a multifactorial progressive degenerative disease of the macula, a part of the retina that is responsible for the central sharp and colored vision (Campagne et al. 2014). The disease is classified into a slowly progressing atrophic form (dry AMD) and a rapidly progressing neovascular form (wet AMD). Whether these two forms are actually separate diseases or one of them progresses to the other one is somewhat controversial (de Jong 2006). While the wet form of AMD affects only 15% of all AMD cases, it accounts for about 90% of AMD-related blindness (Jager et al. 2008).

Figure 1. A cartoon illustration of the different retinal layers in the normal eye (A) and the major characteristics of choroidal neovascularization (CNV) that occur in those layers in the eyes of wet AMD patients (B). RPE; retinal pigment epithelium.
C.1. AMD Risk Factors

Various risk factors predispose to the pathogenesis of AMD such as age, genetic factors and a number of environmental, behavioral and disease conditions that may influence the cellular process underlying the development and progression of all forms of AMD. Older age is the strongest risk factor for the development and progression of AMD, with about 30% prevalence among people 75 years or older (Jager et al. 2008). Advanced AMD has been shown to be more common in Caucasians than in blacks. Hispanics and Asians seem to have a higher prevalence of AMD than blacks but lower than Caucasians (Yonekawa and Kim 2015).

Some genetic factors have been shown to play a role in the development of AMD. A single nucleotide polymorphism (Tyr402His) in the complement factor H (CFH) gene could account for about 50% of the inheritance of AMD (Whitcup et al. 2013). CFH, a major inhibitor of the complement system, is synthesized in the macula and has been shown to be present in extracellular accumulations of lipid deposits called drusen (Hageman et al. 2005). This complement phenotype might result in overreaction to injury and debris in the retina that accumulate with age. Additionally, animal studies have shown that complement activation fosters the production of VEGF, which is strongly implicated in the development of CNV (Bora et al. 2006). Other polymorphisms in complement-related genes, such as factor B (CFB) and C2 genes have also been associated with increased risk of AMD (Heurich et al. 2011). The involvement of non-complement related genes
has been also documented; a polymorphism in the age-related maculopathy susceptibility 2 gene (ARMS2) has been strongly implicated in the development of AMD, independent from CFH (Andreoli et al. 2009). Genetic susceptibility can be influenced by modifiable risk factors such as smoking, obesity, high fat diet, low intake of antioxidants and hypertension (Nowak 2014, Jager et al. 2008). Together these factors are highly predictive of the onset and progression of AMD.

C.2. Mechanisms of Wet AMD

In many mammalian species, the outer segment of the retina, containing photoreceptors, and the retinal pigment epithelium (RPE), depend mainly on the blood supply provided by the choroid. A layer of densely arranged fenestrated capillaries called the choriocapillaris is located underneath the retina and separated from the RPE by a thin elastic membrane called Bruch's membrane (Zarbin 2004). The RPE is a part of the blood-retinal barrier and has several functions such as photoreceptor phagocytosis, participation in the visual cycle and the secretion of growth factors and cytokines that are important for the formation and maintenance of choriocapillaris (Strauss 2005). Researchers have shown that the choroid has the highest blood flow per unit time and weight among other ocular or non-ocular tissues examined (Alm et al. 1973, Alm and Bill 1973), with a maximal flow at the macula. Despite this high blood flow, the human macular choriocapillaris is estimated to have only 11% pericyte coverage, as opposed to 94% for retinal capillaries (Chan-Ling et al. 2011). These features
of the choroidal vessels may render them prone to stress-induced structural changes.

Age-related biological changes take place in the eye, which may contribute to the pathogenesis of AMD in predisposed individuals. With age, drusen are formed between the RPE and Bruch’s membrane (Jager et al. 2008), which is considered a clinical hallmark of AMD. Increased oxidative stress, RPE dysfunction, accumulation of undegradable products of photoreceptor metabolism, increased thickness of Bruch’s membrane, and a decline in choriocapillaris density and lumen diameter are important events that seem to increase linearly with aging. These age-related changes may be associated with the deficiency of nutrients/debris exchange between choriocapillaris and the subretinal space resulting in a further decline in RPE and photoreceptor function (Zarbin 2004). Given that the highest density of photoreceptors is in the macular area (Curcio et al. 1990), it is not surprising that the macula is the most affected area of the eye by those age-related changes.

Wet AMD involves aging changes plus additional pathological processes. The exact mechanism by which CNV formation is initiated is still under debate. However, the role of inflammation in triggering the abnormal growth of choroidal vasculature is currently the most widely accepted model (Tezel et al. 2004, Zarbin 2004). In wet AMD, RPE dysfunction and choriocapillaris injury elicit a chronic inflammatory response in Bruch’s membrane and the choroid. RPE injury
and inflammation foster the production of abnormal ECM, derived mainly from RPE and photoreceptors, which in turn results in further damage in the retina, RPE and the choroid (Zarbin 2004, Campochiaro 2000). Disturbances in RPE – choriocapillaris homeostasis mediate CNV growth from the choriocapillaris through breaks in Bruch’s membrane and the CNV expands underneath the retina or between the RPE and photoreceptors (Yonekawa and Kim 2015). The new vasculature is fragile with increased vascular permeability, which causes hemorrhage, fluid exudation and accumulation of protein and lipid deposits (de Jong 2006, Hageman et al. 2001). This might be accompanied by the formation of fibrotic scarring and the detachment of RPE and eventually irreversible blindness if left untreated.

D. VEGF and Existing Antiangiogenic Drug Therapies

The VEGF is a family of homodimeric proteins that consist of VEGF-A, VEGF-B, VEGF-C, VEGF-D, and placenta growth factor (PIGF). These have a wide range of tissue distribution and functions (Cross et al. 2003). The VEGFs bind to three different receptor tyrosine kinases: VEGFR1, VEGFR2, and VEGFR3 (Cross et al. 2003). VEGF-A, the primary member of the VEGF family, is the master regulator of neovascularization. VEGF-A exists in four different isoforms (121, 165, 189, 206), with VEGF-A165 being the most frequently expressed isoform in normal tissues and in tumors (Ferrara and Adamis 2016). Interestingly, VEGF165b is an additional splice variant of VEGF-A that has been shown to have an inhibitory effect on VEGF-signaling pathway in vitro and in vivo
(Woolard et al. 2004), suggesting that VEGF splicing regulates the switch between pro and antiangiogenic phenotypes. The endothelial-specific receptor VEGFR2 is the main mediator of VEGF-A’s roles in cell proliferation and angiogenesis. Binding of VEGF-A to VEGFR2 leads to receptor dimerization and autophosphorylation resulting in the activation of multiple downstream signaling cascades involved in cell proliferation, migration, ECM degradation and angiogenesis (Munoz-Chapuli et al. 2004).

VEGF expression by different cells, such as Müller cells and RPE in the retina is pivotal for its physiological functions, mainly the formation and maintenance of the retinal and choroidal vasculature (Campochiaro 2000). Apart from vasculature development, VEGF exerts protective functions on retinal cells and is involved in neural retinal development and neurogenesis. The role of increased expression of angiogenic cytokines (mainly VEGF) has been extensively shown to be associated with the pathogenesis of wet AMD (Ishibashi et al. 1997, Kwak et al. 2000). However, the exact mechanism by which VEGF stimulates CNV formation is not clear, and whether it is only necessary or also sufficient to generate CNV is still controversial (Oshima et al. 2004, Kvanta 2006). Therapeutic approaches currently available for wet AMD aim at sealing off the leaky vasculature using laser photocoagulation and/or photodynamic therapy as well as inhibiting new vessel formation (Dorrell et al. 2007). Several anti-VEGF drugs have been recently used such as pegaptanib (Macugen®, Valeant), bevacizumab (Avastin®, Genentech), ranibizumab (Lucentis®, Genentech) and
pugans to VEGF-A\textsubscript{165}, the isoform primarily responsible for pathological ocular angiogenesis (Ng et al. 2006). Bevacizumab is a humanized monoclonal antibody to VEGF, inhibiting VEGF-receptor interaction (Gunther and Altaweel 2009). Ranibizumab is a recombinant humanized fraction of anti-VEGF antibody that binds to all VEGF isoforms (Rosenfeld et al. 2006). Afiblercept, known as VEGF Trap, is a fusion protein that consists of VEGF receptor-binding sequences fused to a segment of a human antibody backbone (Stewart 2012a).

These drugs all act by targeting the VEGF signaling pathway at the level of ligand-receptor interaction. They have been shown to be successful in many wet AMD patients. Meanwhile, they are still under investigation for their potential therapeutic effect on ROP and DR (Andreoli and Miller 2007). However, over 30\% of AMD patients remain unresponsive (Lux et al. 2007). As biologics, these drugs have an unfavorable cost to benefit ratio (Mitchell et al. 2011). Moreover, since VEGF signaling is also required for the survival of quiescent endothelial cells and glial cells that nourish endothelial cells in almost all the tissues of the body, these drugs can cause significant systemic side effects such as myocardial infarction, stroke, delayed wound healing and non-ocular hemorrhage even when the drugs are administered intravitreously (Stewart 2012b). Additionally, several ocular side effects can be associated with intravitreous injections of anti-VEGF drugs, such as intraocular inflammation, ocular hemorrhage, and retinal detachment (Falavarjani and Nguyen 2013). Therefore, there is a strong need to
develop new, affordable drugs specifically targeted for ocular angiogenesis with minimal side effects to complement and perhaps combine with existing therapies.

**E. The Laser-induced Choroidal Neovascularization Mouse Model (L-CNV)**

The development of animal models for wet AMD is crucial for the understanding of the biology and molecular mechanisms of the disease as well as screening for new drugs. As mentioned earlier, the formation of CNV involves three major components; inflammation, angiogenesis, and proteolysis. The in vivo exploration of wet AMD currently involves several mouse models that recapitulate a single or multiple aspects of the disease pathology (Grossniklaus et al. 2010). In addition to mimicking the pathology of human disease, animal models need to be efficient, stable and reproducible. To date, there is no ideal in vivo model that recapitulates the complex pathology of wet AMD. Several genetically engineered animal models have been developed for the study of different factors of wet AMD such as the complement factor pathway models (e.g. *cfh* knockout mice, transgenic *cfh* Tyr402His mice), oxidative damage models (e.g. superoxide dismutase 2 (SOD2) knockdown mice), and lipid metabolism models (e.g. apolipoprotein E (apoE) knockout mice) (Pennesi et al. 2012). While these models are important for elucidating the role of these mediators in the development and progression of AMD, they do not involve CNV formation (Pennesi et al. 2012). Other models such as transgenic mice overexpressing VEGF164 in the RPE, and apoE transgenic mice have been shown to spontaneously develop CNV. Disadvantages of these models are
mainly related to the length of time required for the development of CNV and that a relatively small percentage of the eyes develop CNV. Therefore, it has become apparent that induced damage of Bruch’s membrane using laser or mechanical injury plays a central role in the consistent development of CNV (Rakoczy et al. 2006).

Using a high-powered laser to induce injury to RPE/Bruch’s membrane complex was initially introduced in non-human primates (Ryan 1979), then rodent adaptations of this model were created (Dobi et al. 1989). The laser-induced choroidal neovascularization (L-CNV) model depends on using focused laser energy to induce a break in Bruch’s membrane, which leads to the formation and growth of new blood vessels from the choroid into the subretinal space (Figure 2). The first mouse model of L-CNV was produced by Campochiaro’s group in 1998, by using high intensity, small spot size and short duration of laser, which was found to reliably produce CNV (Tobe et al. 1998). Three laser burns were introduced to the posterior pole of the eye, with a higher rate of CNV formation seen in burns with acute bubble formation, indicative of the rupture of Bruch’s membrane.

Numerous methods have been described to analyze experimental CNV. Fluorescein angiography is used for qualitative visualization of leakage from CNV lesions, with some limitations, including that mature lesions might not leak possibly due to the surrounding RPE (Tobe et al. 1998). Histology and
immunohistochemistry could be used to identify CNV. However, it requires labor-intensive work and it is difficult to quantify. RPE/choroid/sclera flatmount preparation and fluorescent staining of endothelial cells, followed by three-dimensional (3D) volumetric analysis using a confocal microscope is now the standard method for CNV lesion analysis as it provides a robust and reliable quantification of lesions' vascular volume (Campos et al. 2006).

Figure 2. The L-CNV mouse model. A. A cartoon showing the steps of laser application and CNV production. B. A fundus photograph showing the three burns produced after laser treatment.

A primary limitation of this model is the artificial nature of CNV production that does not mimic the complexity of human pathology of wet AMD. In addition to the fact that mice do not have a defined macula, this model represents a wound-healing mechanism following injury at the level of Bruch's membrane, which depends mainly on inflammation; different from wet AMD in which genetic susceptibility plays a major role (Campochiaro 2010). Currently, the L-CNV model is widely accepted and the most commonly used model for CNV as it
recapitulates the biological processes involves in CNV formation in wet AMD such as inflammation and angiogenesis (Lambert et al. 2013). The CNV formation in this model is robust, consistent, reproducible, and results in CNV in approximately 80% of the lesions. Additionally, the L-CNV model is considered the model of choice for testing the efficacy of new drugs either through systemic or intraocular routes of administration. This model has been used for preclinical evaluation of the therapeutic effects of drugs in patients with wet AMD, such as with the “VEGF-trap”, aflibercept (Saishin et al. 2003, Heier et al. 2012).

F. Natural Product Inhibitors of Ocular Angiogenesis

Exploring natural compounds for the development of novel therapies for multiple diseases including wet AMD has been approached in several studies (Kim, Kim, Yu, Park, et al. 2008, Kim, Kim, Yu, Jun, et al. 2008). The medicinal use of natural compounds derived from plants, animals and microorganisms was introduced in ancient medicine thousands of years ago (Ji et al. 2009). Natural products served as the sole pharmacological source for the treatment of diseases for most of human history, where some herbs were chewed to relieve pain, and others were applied topically on wounds to improve healing. Screening natural products to provide novel human therapeutics was maximized by the Western pharmaceutical industry resulting in a broad spectrum of pharmaceuticals from non-synthetic molecules (Newman et al. 2003). In the 19th century, the development of analytical and structural chemistry provided the tools to purify different compounds from natural sources and to determine their
structures, which in turn, provided information about their possible molecular targets in the human body (Ji et al. 2009). In the 20th century, about 80% of the approved drugs were natural products or analogues derived from them. Natural source-derived antibiotics such as the penicillins, immunosuppressants for organ transplants such as cyclosporine, and anticancer drugs such as taxols revolutionized medicine and improved quality of life (Li and Vederas 2009). The functions and structures of natural products and their molecular targets are believed to have evolved to interact with one another (Schmidt et al. 2007), suggesting that natural products might serve as optimal small molecule ligands for some human targets.

Despite these advantages, a decline in natural products-based drug discovery has been experienced in the past decades with the advent of molecular biology and rational, structure-based design that made it possible to design synthetic chemicals to target specific proteins. However, the past few years have witnessed a resurgence of interest in the use of natural compounds as a basis for drug development, with several promising compounds having undergone clinical evaluation for the treatment of varied conditions such as neoplastic, immunological, and inflammatory diseases (Mishra and Tiwari 2011). Interest in natural products will continue for many reasons: they are a matchless source of novel drug leads and inspiration for the synthesis of synthetic or semi-synthetic molecules (Mishra and Tiwari 2011), they can work synergistically to potentiate the activity of other drugs and overcome drug resistance (Schmidt et
al. 2007), and they can offer powerful leads with favorable absorption, distribution, metabolism, excretion, and toxicity (ADMET) characteristics (Corson and Crews 2007). Advances in separation and structure determination technologies, along with the ability to modulate biological activity through structural modifications, have made a wide variety of natural products and derivatives readily available (Koehn and Carter 2005).

Numerous natural compounds have been tested as inhibitors of uncontrolled angiogenesis in various pathological conditions, such as cancer and inflammation, and also in blinding eye diseases. The concept of angiogenesis as an important therapeutic target was initially introduced due to its involvement in tumor growth and metastasis (Folkman 1995). Pathological ocular neovascularization has a significant contribution to ROP, PDR, AMD, as well as other ocular diseases (Zhang and Ma 2007).

A select subset of natural compounds, spanning a variety of compound classes, have 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. They have long been recognized for their antioxidant properties (Manach et al. 2004). Therefore, they have been tested for their potential therapeutic effects in many diseases such as cancer and inflammatory and cardiovascular diseases. Polyphenols are 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 3). Many flavonoids have been studied for their beneficial roles in ocular diseases (Majumdar and Srirangam 2010, Sulaiman et al. 2014).
Figure 3. Chemical structures of antiangiogenic natural products tested in the context of ocular neovascular diseases.
G. Natural Products in Ocular Neovascularization Models

G.1. In Vitro Models of Angiogenesis

Extensive screening of the antiangiogenic activity of novel compounds in vitro and in vivo is crucial to investigate their possible potential for the treatment of angiogenic diseases. In vitro assays for angiogenesis utilize cultured endothelial cells to test the effects of the compounds on cell proliferation, migration and tube formation. Endothelial cell proliferation can be investigated by multiple assays, such as alamarBlue (O'Brien et al. 2000), MTT (Denizot and Lang 1986) or EdU incorporation assays (Buck et al. 2008). Migration of endothelial cells can be evaluated in vitro by techniques such as the scratch wound migration assay (Liang et al. 2007). Tube formation is one of the most common tests for angiogenesis, which measures the ability of endothelial cells to form three-dimensional structures (tubes) (Madri et al. 1988).

Human umbilical vein endothelial cells (HUVECs) and bovine aortic endothelial cells (BAECs) are established cell systems. These in vitro systems provide a rapid, practical and reproducible way for screening of large numbers of compounds. However, for screening of compounds specifically for ocular angiogenesis, it is important to use more relevant, tissue specific endothelial cells; human retinal microvascular endothelial cells (HRECs) are a popular choice in this case, and are commercially available. Nearly all the natural compounds shown in Figure 3 were effective in angiogenesis inhibition in vitro, evaluated by measuring the compound concentration that induces 50% maximal
inhibition of cell growth (GI$_{50}$). The majority of them were tested in HUVECs, a model endothelial cell line, but not from the same vascular bed as those endothelial cells implicated in ocular diseases. Withaferin A (14) was the most potent in inhibiting proliferation with GI$_{50}$ = 12 nM (Mohan et al. 2004). Genistein (3) and quercetin (6) showed equal potencies in inhibiting BAEC proliferation at 20 µM concentration (Koroma and de Juan 1994, Jackson and Venema 2006). Luteolin (1), hesperetin (4), cremastranone (5) and synthetic derivatives, curcumin (9), and decursin (13) were tested in the most disease-relevant cells, HRECs. SH-11037, a synthetic derivative of cremastranone (5), showed the highest potency in these cells, compared to other compounds, with GI$_{50}$ 55 nM, and with 10-fold selectivity over HUVECs (Basavarajappa HD 2014).

G.2. In Vivo Models

G.2.1. Proliferative DR

Currently, there is no perfect model for proliferative DR; the most commonly used animal model for diabetic retinopathy is induced by intraperitoneal (i.p.) administration of streptozotocin (STZ) in mice and rats (Jo et al. 2013), but these animals do not develop neovascularization. The hyperglycemic action of STZ induces diabetes and the development of retinopathy later with the disease progression. Many natural compounds including genistein (3), hesperetin (4), curcumin (9), resveratrol (10), and decursin (13) demonstrated potential in ameliorating retinopathy in the STZ model after oral administration. Those effects were due to inhibiting retinal
oxidative stress and/or increasing antioxidant defense systems as seen with hesperetin (4), and curcumin (9) (Kumar et al. 2013, Kowluru and Kanwar 2007), or due to suppressing the production of pro-inflammatory mediators and growth factors as in the case of resveratrol (10), and decursin (13) (Soufi et al. 2012, Yang et al. 2013). Meanwhile, combretastatin A4 (12) failed to show an effect on long-term galactose fed dogs, a different preclinical model for DR where neovascularization slowly develops over a period of months to years as in clinical cases (Kador et al. 2007).

G.2.2. ROP

Oxygen-induced retinopathy (OIR) is the major model in use for study and evaluation of pathological angiogenesis resulting from ischemia such as retinal neovascularization (Smith et al. 1994). In this model, early postnatal animals (P7) are exposed to hyperoxia during early retinal development and remain in the oxygen chamber for 5 days (to P12). Hyperoxia results in suppression of oxygen-regulated factors such as HIF-1α and its downstream VEGF, which in turn causes cessation of existing retinal blood vessels and new vessel growth (Hellstrom et al. 2013). The increased metabolic activity of the poorly vascularized retina results in the activation of hypoxia-driven VEGF expression upon return to room air. Neovascularization of leaky vessels is noticed within about 5 days (P17), which may result in fibrous scarring and retinal detachment. The OIR model mimics the pathological characteristics of ROP, with a consistent and reproducible angiogenic response; therefore, it has become important in
studying the disease mechanisms and investigating potential treatments for ROP (Smith et al. 1994). Several natural compounds demonstrated antiangiogenic effects in the OIR model with different potencies suggesting their potential in the treatment of ROP. Luteolin (1) and deguelin (8) showed equal potencies in suppressing retinal neovascularization after intravitreal injection of 0.1 µM compound concentration (Park et al. 2012, Kim, Kim, Yu, Shin, et al. 2008). Meanwhile, genistein (3), resveratrol (10), and honokiol (11) were effective after systemic i.p. administration (Wang et al. 2005, Kim and Suh 2010, Vavilala et al. 2013).

G.2.3. Wet AMD

Several natural compounds such as apigenin (2), genistein (3), cremastranone (5), quercetin (6), isoliquiritigenin (7), deguelin (8), and combretastatin A4 (12) showed a significant reduction in neovascularization after laser photocoagulation. Deguelin (8) was the most potent in inhibiting neovascularization after intravitreal injection at 0.1 µM concentration (Kim, Kim, Yu, Park, et al. 2008). Cremastranone (5) was of moderate potency at 1 µM concentration (Kim, Kim, Yu, Jun, et al. 2008), compared to isoliquiritigenin (7), which was effective in the 10-200 µM range (Jhanji et al. 2011). Apigenin (2) and combretastatin A4 (12) suppressed CNV formation when administered systemically (Zou and Chiou 2006, Nambu et al. 2003). Genistein (3) showed partial inhibition of CNV formation, compared to specific inhibitors of VEGF/PEDF receptor kinases, when given systemically (Kwak et al. 2000). While systemic
drug administration is favored over the intravitreal route, it is influenced by different challenges, such as the blood-retinal barrier, that will control the effectiveness of the drug reaching the eye (Macha and Mitra 2003). On the other hand, the intravitreal route is more effective in delivering maximum concentration of the drug to the eye with minimal systemic side effects; therefore, it is the most commonly used for treatments of ocular neovascular diseases.

H. Mechanisms of Angiogenesis Inhibition by Natural Products

Identification of the mechanisms of action of antiangiogenic compounds is a crucial step for them to proceed to clinical trials. Most of the natural compounds under investigation have been tested for their effect on known angiogenic pathways (Figure 4). However, elucidating novel pathways for angiogenesis inhibition is vital to overcome the resistance that might emerge with long-term administration of angiogenic inhibitors (Kerbel and Folkman 2002).

Apart from VEGF, the most dominant angiogenic factor in neovascularization, other growth factors such as fibroblast growth factor (FGF) and angiogenin have also been shown to promote angiogenesis (Qazi et al. 2009). On the other hand, there are several endogenous antiangiogenic factors such as PEDF and angiostatin that are required to maintain homeostasis of angiogenesis (Nyberg et al. 2005). The balance between endogenous pro- and antiangiogenic factors tightly regulates homeostasis of ocular vasculature. When this balance is disturbed, formation of new blood vessels occurs, which is
implicated in the diseases discussed above. Under certain conditions, the production of angiogenic stimuli causes endothelial cells to secrete proteases such as matrix metalloproteases (MMP) and collagenases, which leads to degradation and remodeling of the extracellular matrix (ECM). Apigenin (2) and isoliquiritigenin (7) were shown to inhibit HUVEC migration and tube formation in vitro by downregulating MMPs (Kim 2003, Kang et al. 2010).

Figure 4. A simplified diagram showing the known locations of natural products' effects on selected angiogenic pathways. Angiogenic factors act on their receptors (red) on endothelial cells to activate various downstream signaling molecules (green) and modulators of gene expression (yellow) to mediate angiogenesis. Several natural products interfere with these angiogenic pathways as indicated in purple.
Proangiogenic stimuli such as VEGF and FGF bind to cell surface receptors and activate downstream signaling cascades to promote migration and proliferation of endothelial cells through the newly formed gaps generating new capillaries through which blood begins to flow. Interference with angiogenesis at the receptor level has been demonstrated by natural compounds such as luteolin (1) and honokiol (11), which inhibited VEGFR2 autophosphorylation and activation in HUVECs (Bagli et al. 2004, Bai et al. 2003), and decursin (13) in HRECs (Yang et al. 2013). Other compounds inhibited the activation of downstream signaling pathways of growth factors, for instance luteolin (1), which inhibited VEGF-induced PI3K/Akt activation in HUVECs (Bagli et al. 2004), and curcumin (9), which blocked VEGF-induced PKCβII translocation in HRECs (Figure 4) (Premanand et al. 2006). Cremastranone (5) inhibited FGF signaling in HUVECs (Shim et al. 2004). Growth factors also increase vascular permeability through stimulation of nitric oxide (NO) synthesis and release from endothelial cells, a step that was inhibited by quercetin (6) and resveratrol (10) (Jackson and Venema 2006, Kim and Suh 2010).

Tumor necrosis factor-α (TNF-α) is a very potent angiogenic stimulator that binds to TNF receptors and activates nuclear factor-kappa B (NF-κB), a transcription factor that is central to the regulation of many genes, such as those encoding adhesion molecules and angiogenic mediators (Grilli et al. 1993). Some natural compounds interfered with NF-κB activation and expression of downstream genes in vitro either by blocking NF-κB nuclear translocation as
seen with SH-11052, a synthetic isomer of cremastranone (5) (Basavarajappa et al. 2014), or as with withaferin A (14) by targeting the ubiquitin proteasome pathway (UPP) which regulates NF-κB activation (Figure 4) (Palombella et al. 1994).

Endothelial cells respond to oxygen, too. Under normal conditions, the oxygen sensor prolyl hydroxylase domain protein (PHD) hydroxylates hypoxia-inducible factor 1α (HIF-1α), which is then targeted for proteasomal degradation. During hypoxia, PHD is inactivated and HIF-1α activates the transcription of multiple genes including growth factors such as VEGF and TNF-α, cytokines such as interleukins (IL-6 and IL-8), proinflammatory enzymes such as cyclooxygenase 2 (COX2), and proteases such as MMPs. This broad transcriptional response increases blood flow and oxygen supply by angiogenesis (Fraisl et al. 2009). However, HIF-1α can be activated under non-hypoxic conditions by growth factors such as VEGF, causing a vicious cycle of uncontrolled neovascularization. Apigenin (2) interfered with HIF-1α signaling in vitro, by initiating HIF-1α degradation and inhibiting the expression of downstream VEGF in HUVECs (Zou and Chiou 2006). Meanwhile, genistein (3) inhibited hypoxia-induced expression of HIF-1α, and deguelin (8) reduced HIF-1α expression and mediated its degradation in vivo in the OIR model (Figure 4) (Wang et al. 2005, Kim, Kim, Yu, Shin, et al. 2008). While deguelin (8) was very potent in suppressing retinal and choroidal neovascularization in the OIR and L-CNV mouse models, respectively, some undesirable cell toxicity has been
demonstrated which could be an obstacle for its clinical use (Kim, Chang, et al. 2008).

A clear characterization of the molecular targets by which the above natural products interfere with angiogenesis is still needed. However, unlike most natural products discussed here, a direct target of withaferin A (14) is known. It binds to the intermediate filament (IF) protein vimentin, which plays a critical role in angiogenesis and cancer growth, causing IF degradation and down-regulation of their expression levels. This leads to attenuation of retinal gliosis associated with several degenerative conditions in the eye including AMD (Bargagna-Mohan et al. 2010, Bargagna-Mohan et al. 2007). Further elucidation of the direct targets of other natural compounds is important to develop more specific and potent analogs with minimal side effects and to test the combination of different compounds targeting distinct angiogenic mechanisms.

I. The Synthetic Homoisoflavonoid, SH-11037

Despite the high number of natural compounds tested for their antiangiogenic potential in multiple diseases such as cancer, only a select subset of them has been tested specifically in the context of ocular angiogenesis. The structural, chemical and pharmacological diversity of homoisoflavonoids have attracted researchers interested in developing small molecule therapies for various diseases (Lin et al. 2014).
Cremastranone is a homoisoflavonoid isolated from the bulb of *Cremastrastrum appendiculata*, which is used in East Asia as a traditional medicine, taken internally to treat several cancers, and applied externally for skin lesions. Cremastranone was previously reported to possess anti-angiogenic activity both in vitro and in vivo and was identified as a potent inhibitor of the proliferation of HUVECs (Shim et al. 2004). Moreover, it inhibited angiogenesis in vitro, with some selectivity for suppressing the proliferation of endothelial cells compared to other ocular cell types. Selectivity for microvascular endothelial cells, representing those in the eye, over macrovascular endothelial cells and non-endothelial cell types is suggestive of a lack of off-target cytotoxicity in vivo (Chapter IV). Cremastranone was shown to inhibit vascular tube formation, migration, and new vessel growth induced by basic fibroblast growth factor. Its anti-angiogenic properties were further confirmed in vivo in the L-CNv and OIR mouse models (Kim, Kim, Yu, et al. 2007, Kim, Yu, et al. 2008), used for testing novel therapies for wet AMD and ROP, respectively. Moreover, injection of cremastranone into the vitreous of normal adult mice showed no short-term cytotoxic or inflammatory effects on the retina, nor did it induce apoptosis of retinal cells (Kim, Yu, et al. 2008). These results suggest that proliferative ocular vascular diseases such as ROP, DR, and AMD may be targeted using cremastranone or its derivatives. Other recent work has suggested that cremastranone has potent anti-inflammatory activity in the context of UV-induced skin inflammation (Hur et al. 2010) and allergy (Lee, Hur, et al. 2014).
Cremastranone was previously shown to modulate the activity of multiple signaling pathways. It induces expression of p21WAF1 (CDKN1A), an inhibitor of the cyclin-dependent kinase Cdc2 (CDK1) (Kim, Kim, Kim, et al. 2007). Cremastranone blocks prostaglandin synthesis from arachidonic acid (Hur et al. 2010, du Toit et al. 2005), and decreases phosphorylation of the mitogen activated protein kinases (MAPK). Additionally, it blocks nuclear translocation of NF-κB and production of inflammatory cytokines (Lee, Hur, et al. 2014, Basavarajappa et al. 2014).

Despite the well-established antiangiogenic activity of cremastranone, other homoisoflavonoids have not been explored for developing potent, endothelial cell specific compounds. Although multiple signaling pathways are modulated by cremastranone, its exact mechanism of action remains unknown, rendering a cell-based analysis of efficacy the most appropriate route for the development of novel derivatives. We developed a structure-activity relationship of homoisoflavonoids for inhibiting angiogenesis, which resulted in identification of potent, microvascular endothelial-cell specific antiangiogenic molecules for lead optimization (Basavarajappa et al. 2015). In collaboration with Seung-Yong Seo’s laboratory in South Korea, using cremastranone as our primary scaffold, we envisioned homoisoflavonoid analog design by which several substituents on the A and B rings could be varied as shown in Figure 5.
After screening over 100 derivatives of cremastranone, modification at the C3′ position revealed that addition of N-carbamate amino acids improved inhibitory activity on HREC proliferation. A more potent synthetic derivative of cremastranone, named SH-11037 (15) (Figure 6) was developed, in which phenylalanyl incorporation showed more potent antiproliferative activity. Moreover, SH-11037 demonstrated a remarkable selectivity of about 10-fold for the disease-relevant HRECs over macrovascular endothelial cells, and negligible effects on other ocular cell types suggesting the absence of off-target cytotoxic effects. With such an interesting potency and selectivity of SH-11037 in vitro, a full evaluation of its antiangiogenic activity in vitro and its therapeutic potential in vivo in animal models of ocular angiogenesis is worthy of investigation.
Figure 6. Structure of SH-11037, a synthetic derivative of cremastranone generated after structure-activity relationship (SAR) study. Modified groups are shown in red.

J. Summary, Hypothesis and Specific Aims

The wet form of AMD accounts for 90% of AMD-related blindness with about 200,000 new case diagnosed every year in the United States alone (Congdon et al. 2004). This disease is characterized by pathological CNV in which aberrant vasculature originates in the choroid and grows through a break in Bruch’s membrane to the sub-retinal space. This break may be secondary to trauma, a degenerative process, and/or tissue inflammation (Grossniklaus and Green 2004). These new blood vessels are fragile and leaky, and their rupture causes hemorrhage and accumulation of fluids resulting in an irreversible damage to retinal function. These vessels may also induce the formation of fibrous scarring causing rapid and permanent vision loss (Ehrlich et al. 2008). Currently, standard treatments targeting VEGF have been successful in slowing down the disease progression in some AMD patients (Prasad et al. 2010). However, about 30% of wet AMD patients remain unresponsive to these therapies (Lux et al. 2007). As biologics, these drugs can also have unfavorable cost to benefit ratio (Mitchell et al. 2011). Additionally, several ocular and
systemic side effects have been associated with intravitreal injections of anti-VEGF drugs such as ocular and non-ocular hemorrhage, myocardial infarction and delayed wound healing (Falavarjani and Nguyen 2013). Therefore, there is a strong need to develop new, affordable drugs with minimal side effects to complement and perhaps combine with existing therapies.

The natural product cremastranone is a promising therapeutic lead for wet AMD (Shim et al. 2004). This potent antiangiogenic compound suppressed HUVEC proliferation, migration and tube formation in vitro at low micromolar concentrations (Kim, Kim, Kim, et al. 2007), with similar effects in HRECs (Lee, Basavarajappa, et al. 2014). It also inhibited in vivo CNV in the laser photocoagulation mouse model, without causing retinal inflammation or apoptosis (Kim, Kim, Yu, Jun, et al. 2008). We identified a novel synthetic derivative of cremastranone, SH-11037, in the course of a structure-activity relationship study of antiangiogenic homoisoflavonoids. SH-11037 is very potent in inhibiting the proliferation of HREC with GI_{50} of 55 nM, with 10 fold selectivity over HUVECs, without inducing apoptosis (Basavarajappa et al. 2015). It also had negligible effects on proliferation of retinoblastoma and uveal melanoma cell lines (Basavarajappa et al. 2015).

The overall objective of the proposed study is to fully characterize the mechanism of SH-11037’s antiangiogenic effects and elucidate its potential in the treatment of CNV. My central hypothesis is that SH-11037 ameliorates
pathological choroidal neovascularization by a novel VEGF-independent mechanism. I tested this hypothesis by pursuing the following Specific Aims:

**Specific Aim I:** Evaluate the antiangiogenic activity of SH-11037 in vitro, ex vivo, and in vivo in CNV-relevant systems

   Based on the potent antiproliferative effect of SH-11037 in vitro, I tested the antiangiogenic activity of SH-11037 in vitro using HRECs to evaluate the DNA synthesis, cell migration, and tube formation in the presence and absence of SH-11037. I evaluated the effect of SH-11037 on the sprouting of choroidal tissues ex vivo using the choroidal sprouting assay. Additionally, I tested SH-11037’s effect in vivo in an animal model of CNV. First, I determined the absence of signs of ocular toxicity of intravitreally injected SH-11037 in mice. Then I examined its efficacy in the laser photocoagulation mouse model in comparison with the standard anti-VEGF therapy.

**Specific Aim II:** Evaluate the VEGF-independent effect of SH-11037 and identify its binding partners.

   Combination treatments of different SH-11037 and anti-VEGF concentrations were tested to examine VEGF-independency and the potential of lowering the anti-VEGF treatment dose using a combination with SH-11037, which might be beneficial in minimizing the adverse effects of anti-VEGF therapies. This was done in vitro by testing the antiproliferative effects of individual and combination treatments on HRECs, and in vivo in the L-CNV mouse model. To find target(s) of SH-11037, I used a photoaffinity pulldown
approach in which a novel affinity reagent was used to isolate SH-11037 binding proteins from HRECs. Then, the role(s) of these target proteins in HRECs were validated and their relevance to angiogenesis was explored in vitro and in vivo.

**K. Dissertation Overview**

This dissertation describes experiments directed towards testing the above specific aims. Chapter II discusses the materials and methods used for the completion of these studies. Chapter III presents the development of a novel quantification method for optical coherence tomography (OCT). This was used throughout the animal experiments to monitor the progression of CNV vascular volume in vivo at different time points. The OCT technology is explained in this chapter, with the importance and challenges of its use in animal studies. Then, the comprehensive evaluation of the antiangiogenic activity of SH-11037 is discussed in Chapter IV. This was done in different CNV-relevant systems going from in vitro using HRECs, to ex vivo in isolated mouse choroidal tissues, and finishing with the in vivo L-CNV mouse model. The detailed evaluation of the safety of SH-11037 in vivo in normal adult mice is also discussed in this chapter. Since the existing therapies for wet AMD target the VEGF pathway and are associated with various side effects, the development of VEGF-independent small molecules is necessary. Therefore in Chapter V, I discuss the evaluation of the VEGF-independent mode of action of SH-11037 and the identification of its binding partners. Finally, Chapter VI emphasizes the major findings of this study, and discusses potential future experiments that might be completed to fully
characterize the possible potential of SH-11037 as a novel small molecule therapy for wet AMD.
CHAPTER II. MATERIALS AND METHODS
A. In Vitro Experiments

A.1. Materials

EBM-2 and IMDM growth media were purchased from Lonza (Walkersville, MD, USA). RPMI and Ham’s/F-10 media were purchased from Thermo Scientific (Waltham, MA, USA). HRECs and Attachment Factor were purchased from Cell Systems (Kirkland, WA, USA). Clonetics® HUVECs and aortic ECs were purchased from Lonza. All endothelial cells were used between passages 5 and 8. Endothelial Growth Medium (EGM-2) was prepared by mixing the contents of an EGM-2 “Bullet Kit” (Cat no. CC-4176) with Endothelial Basal Medium (EBM) (Lonza). The EGM-2 “Bullet Kit” contains hydrocortisone, human fibroblast growth factor (hFGF), VEGF, R3-insulin like growth factor (R3-IGF-1), ascorbic acid, human epidermal growth factor (hEGF), gentamycin and heparin along with 2% fetal bovine serum (FBS). 92-1 cells (De Waard-Siebinga et al. 1995) were grown in RPMI medium containing 10% FBS and 1% penicillin-streptomycin (pen-strep). Y79 cells (Reid et al. 1974) were grown in RB medium (IMDM +10% FBS+ 55 µM β-mercaptoethanol + 10 µg/mL Insulin + 1% pen-strep). ARPE19 cells were grown in Ham’s/F-10 + 10% FBS + 1% pen-strep. Hepatocellular carcinoma cell line HepG2 was grown in IMDM medium containing 10% FBS and 1% pen-strep. SH-11037 (15) and SH-11098 (16) were synthesized by collaborator S.Y. Seo’s laboratory as previously reported (Basavarajappa et al. 2015). The synthesis of affinity reagents AR1 (17) and AR2 (18), and negative control reagent, NC (19), will be reported elsewhere (Lee 2016). Monoclonal antibodies against α-tubulin (DM1A) and β-actin (AC40) were
purchased from Sigma-Aldrich (St. Louis, MO, USA). The primary antibody against porcine sEH (Y-13) and antibody against human and mouse sEH (H-215), and p38 MAPK antibody were obtained from Santa Cruz (Dallas, TX). Antibodies against GFAP (D1F4Q) and cleaved caspase 3 (D175), VEGFR2, phospho-VEGFR2 (Tyr 1175) (19A10), phospho-p38 (Thr180, Tyr182), ERK1/2, phospho-ERK1/2 (Tyr204) and IkB antibodies were from Cell Signaling (Danvers, MA). MCP1 antibodies were from Novus Biologicals (Littleton, CO). Secondary antibodies were from Thermo Fisher Scientific (Pittsburgh, PA, USA). The Trizol, TaqMan probes, and qPCR master mix were procured from Life Technologies (Carlsbad, CA). The iScript reverse transcriptase was from Bio-Rad (Hercules, CA).

### A.2. Cell Proliferation Assay

The proliferation of cells was monitored by an alamarBlue based fluorescence assay as described previously (Basavarajappa et al. 2014). Four cell types were used: HRECs, HUVECs, 92-1, and Y79. Briefly, 2,500 cells in 100 µL growth medium were incubated in 96-well clear bottom black plates for 24 hours followed by 48 hours’ incubation with different concentrations of each test compound (range: 0.5 nM to 500 µM). At the end of the incubation, 11.1 µL of alamarBlue reagent (AbD Serotec, Kidlington, UK) was added and 4 hours after, fluorescence readings were taken with excitation and emission wavelengths of 560 nm and 590 nm respectively. Data were analyzed and dose response curves generated using GraphPad Prism software (v. 6.0).
A.3. EdU Incorporation Assay

The assay was carried out as described before (Basavarajappa et al. 2014). HRECs (25,000) were seeded onto coverslips coated with attachment factor and grown for 24 hours before starving in serum-free EBM-2 medium. After starvation for 12 hours, the cells were incubated with 10 µM EdU in the presence of various concentrations of SH-11037 for 8 hours. Then the cells were processed according to the manufacturer’s instructions for the click-iT EdU assay kit (Life Technologies, Grand Island, NY, USA). The images were taken using an EVOS-fl digital microscope (AMG, Mill Creek, WA, USA) and data were analyzed using ImageJ software v. 1.48v (http://imagej.nih.gov/ij/).

A.4. Apoptosis Assays

Caspase-3 immunofluorescence assay: The assay was carried out as described previously (Basavarajappa et al. 2014). Briefly, cells were plated on coated coverslips and incubated in EGM-2 medium overnight before treating with indicated concentrations of SH-11037. After 4 hours of compound treatment the cells were fixed in 4% paraformaldehyde and permeabilized using 0.5% Triton X-100 solutions prepared in PBS. Then cells were immunostained using antibody against activated caspase 3 and imaged using an LSM 700 confocal microscope (Zeiss, Thornwood, NY, USA).

TUNEL assay: Cells (25,000 per coverslip) were seeded on each coverslip coated with attachment factor in a 6-well plate and grown overnight in EGM-2
medium. Next day, fresh medium with the indicated concentrations of SH-11037 was added to cells and they were incubated for 4 hours. Cells were then fixed in 4% paraformaldehyde prepared in PBS for 20 minutes at room temperature. The coverslips were washed twice in PBS and incubated further for 20 min in 0.25% Triton X-100 in PBS. Then coverslips were washed in PBS twice and apoptotic cells were visualized using the Click-iT TUNEL Alexa Fluor-594 imaging assay kit (Cat no. C10246, Molecular Probes, Eugene, OR, USA), as per the manufacturer's instructions, with DAPI counter-stain. The percentage of apoptotic cells was counted on three low-power fields per coverslip using Image J software and analysed using GraphPad Prism software (v. 6.0).

A.5. Scratch-Wound Assay

HRECs (10^5) were seeded in each well of a 6-well plate coated with attachment factor. The cells were incubated in EGM-2 medium until confluent (~24 hours). The cells were then starved for 12 hours in serum free EBM-2 medium. After starvation, a straight scratch was introduced in the well with a sterile fine 10 µL micropipette tip. The well was rinsed twice with EBM-2 medium to remove unbound cells and debris. Then cells were incubated in EGM-2 medium in the presence of the indicated concentrations of SH-11037 at 37°C and 5% CO₂. After 8 hours, images were taken using the EVOS microscope and the number of migrated cells into the scratched area was counted.
A.6. Tube Formation Assay

A Matrigel based tube formation assay was performed to monitor the tube-formation ability of HRECs in the presence of SH-11037 as described previously (Basavarajappa et al. 2014). Briefly, 7,500 cells in 100 µL EGM-2 medium were incubated in the presence or absence of SH-11037 in 96-well clear plates coated with 50 µL of Matrigel basement membrane. After 8 hours, the images were recorded using the EVOS microscope, polygons formed were manually counted, and the tube length was measured using angiogenesis analyser macros in ImageJ (http://image.bio.methods.free.fr/ImageJ/?Angiogenesis-Analyzer-for-ImageJ).

A.7. Preparation of Photoaffinity Reagents

For pulldowns, Neutravidin agarose beads (1 mL of 50 % slurry) were washed three times in buffer A containing 25 mM Tris-HCl pH 7.4, 150 mM NaCl, 2.5 mM sodium pyrophosphate, 1 mM phenylmethylsulfonyl fluoride (PMSF), 0.1 mM sodium orthovanadate, 10 µg/mL aprotinin and 10 µg/mL leupeptin. The beads were then incubated with 75 µM affinity reagents, AR1 (17) and AR2 (18) or negative control reagents, NC (19) overnight at 4°C with rotation. The beads were blocked using 1 mM biotin solution prepared in buffer A for 1 hour followed by incubation with 1 mg/mL cytochrome c solution for 1 hour at 4°C. The beads were then washed three times with buffer A and resuspended in 1 mL.
A.8. Photoaffinity Pulldown Experiments

Flash-frozen porcine brain (20 g) obtained from the Purdue-Indiana University School of Medicine Comparative Medicine Program was homogenized in 50 mL buffer A using a tissue homogenizer. The homogenate was centrifuged at 2000×g for 5 minutes. The supernatant (S1) was then dounced 50 times followed by 10 min sonication with amplitude of 60% in cycles of 10 sec sonication on and 40 sec sonication off (Q125 from QSonica, Newtown, CT, USA). The lysate was then centrifuged at 11,000xg for 30 min. The resulting supernatant (S2) was collected. The pellet (P1) was resuspended in buffer A and then dounced 25 times and centrifuged at 11,000xg for 30 min; supernatant (S3) was collected. Both S2 and S3 supernatants were divided equally and each fraction was incubated with 500 µL photoaffinity or control reagent conjugated to Neutravidin beads for 75 min at 4°C with shaking.

The beads were collected by centrifugation at 500×g for 5 min, then resuspended in 1 mL of buffer B (buffer A + 1% Triton X-100) and irradiated with 365 nm UV light (Mercury bulb H44GS100 from Sylvania in a Blak-Ray 100A long-wave UV lamp) in a 60 mm Petri dish for 30 min at 4°C. The beads were then washed two times in buffer B, followed by three washes in high-salt buffer containing 25 mM Tris-HCl pH 7.4, 350 mM NaCl, 1% Triton X-100 and 1 mM PMSF. The beads were then washed again in salt-free buffer containing 25 mM Tris-HCl, 1% Triton X-100 and 1 mM PMSF. After 5 min incubation, the beads were collected and residual liquid was removed using a Hamilton syringe. The
Neutravidin beads were then boiled in 300 µL of 2× SDS-PAGE gel loading dye containing 30 µL of 2-mercaptoethanol for 10 min at 70°C to release the bound proteins. After boiling, the contents were allowed to cool and after a quick spin the eluate was collected using a Hamilton syringe. The eluates were then analyzed in 4–20% gradient SDS-PAGE and the protein bands were visualized using silver staining (Corson et al. 2011). The protein bands pulled down specifically by photo-affinity reagent were excised from the silver stained SDS-PAGE gel and analyzed by mass spectrometry (IUSM Proteomics Core). Using Sequest™ algorithms and the swine database (UniProt), the identities of the pulled down proteins were confirmed.

A.9. Immunoblot Assay

Cell lysates were prepared by incubating the cells for 10 minutes at 4°C in NP-40 lysis buffer (25 mM HEPES pH 7.6, 150 mM NaCl, 1% NP-40, 10% glycerol, 1 mM sodium orthovanadate, 10 mM NaF, 1 mM PMSF, 10 µg/mL aprotinin, 1 µM pepstatin, 1 µM leupeptin) and then centrifuged at 12,000×g for 15 min at 4°C. Supernatant was collected and protein concentration was determined using a Bradford assay. Equal amounts of total protein (40 µg) from each sample were resolved by 10% SDS-PAGE and then transferred onto PVDF membranes. Proteins were immunoblotted with antibodies against sEH (1:1000 dilution), α-tubulin (1:1000), phospho-VEGFR2 (1:500), VEGFR2 (1:500), β-actin (1:1000). All of the dilutions were made in Tris Buffered Saline-0.05% Tween-20 buffer containing 2% bovine serum albumin (BSA).
A.10. In Vitro Synergy Testing and Analysis

The proliferation of HRECs was monitored by an alamarBlue fluorescence assay as described previously (Lee, Basavarajappa, et al. 2014). Briefly, 2500 cells in 100 µL EBM in the presence of 50 ng/mL recombinant human VEGF-165 (Biolegend, San Diego, CA), were incubated in 96-well clear bottom black plates for 24 hours followed by 48 hours incubation with either 0.5 µM SH-11037, different concentrations of aflibercept (Eylea, Regeneron) (50, 200, 400, 800 µg/mL), or combination treatments. At the end of the incubation, 11.1 µL of alamarBlue reagent was added and 4 hours after, fluorescent readings were taken on a Synergy H1 plate reader (Biotek, Winooski, VT) with excitation and emission wavelengths of 560 nm and 590 nm respectively. Data were analyzed using GraphPad Prism software (v. 6.0).

Synergy analysis was performed as previously described (Borisy et al. 2003). The excess inhibition over the highest single agent (HSA) represents the inhibition of the combination mixture over the highest effect seen with either single agent alone at the same concentration as in the mixture. Bliss additivity calculates the predicted combined response C for two single compounds with effects A and B as: \( C = A + B - A \times B \). The predicted effect C was then subtracted from the experimentally observed percent inhibition to generate excess over Bliss values, where positive values indicate synergistic effects.
A.11. Soluble Epoxide Hydrolase Activity Assay

Small molecule inhibition of soluble epoxide hydrolase (sEH) enzymatic activity was evaluated using an sEH inhibitor screening assay kit (Cayman Chemical, Ann Arbor, MI) following manufacturer’s instructions. Briefly, different concentrations of SH-11037 and \textit{trans}-4-(4-[3-adamantan-1-yl-ureido]-cyclohexyloxy)-benzoic acid (\textit{t}-AUCB) (20) (Cayman Chemical, Ann Arbor, MI) (a specific sEH inhibitor) were tested in the presence of (3-phenyl-oxiranyl)-acetic acid cyano-(6-methoxy-naphthalen-2-yl)-methyl ester (PHOME) as an enzyme substrate. Hydrolysis of PHOME by epoxide hydrolase produces the fluorescent 6-methoxy-2-naphthaldehyde which can be analyzed. Fluorescent readings were taken on a Synergy H1 plate reader (Biotek, Winooski, VT) with excitation and emission wavelengths of 330 nm and 465 nm respectively. Data were analyzed using GraphPad Prism software (v. 6.0).

A.12. qRT-PCR

RNA was extracted from cells using Trizol. cDNA was synthesized from 300 ng RNA using random primers and iScript reverse transcriptase. qPCR was performed in 10 μL volumes in a 384-well plate, with Fast Advanced Master Mix and TaqMan probes on a ViiA7 thermal cycler. Primer/probesets used were: \textit{EPHX2} (Hs00157403_m1), and housekeeping controls \textit{GAPDH} (Hs99999905_m1), and \textit{TBP} (Hs00427620_m1). The data were analysed using the $\Delta\Delta C_t$ method. The expression levels of genes were normalized to the 2 housekeeping genes and calibrated to the HepG2 cells.
B. Ex Vivo Experiments

B.1. Mice

All mouse experiments followed the guidelines of the Association for Research in Vision and Ophthalmology Statement for the Use of Animals in Ophthalmic and Visual Research and were approved by the Indiana University School of Medicine Institutional Animal Care and Use Committee. Wild-type female C57BL/6J mice, 6–8 weeks of age, were purchased from the Jackson Laboratory (Bar Harbor, ME). Mice were housed on a 12 h:12 h light:dark cycle (lights on at 07:00) with access to food and water ad libitum. Intraperitoneal injections of 17.5 mg/kg ketamine hydrochloride and 2.5 mg/kg xylazine mixture were used for anesthesia. At the end of the experiment, mice were euthanized by carbon dioxide asphyxiatioxation followed by cervical dislocation.

B.2. Choroidal Sprouting Assay

Sprouting of choroidal layers was tested as previously described (Shao et al. 2013). Briefly, eyes of 6-8 week old C57BL/6J mice were enucleated immediately after euthanasia. Peripheral parts of the choroid/sclera layer were separated and cut into pieces. Choroid fragments were then placed on growth factor-reduced Matrigel™ (30 µL/well; BD Biosciences, San Jose, CA) in 24 well plates. The plates were then incubated at 37°C for about 10 minutes to allow the Matrigel to solidify. Penicillin-streptomycin and 2.5 mg/mL Plasmocin were added to the EGM-2 medium used for this assay. Medium (500 µL) was added to each well of the plate and incubated at 37°C with 5% CO₂ for 72 hours before any
treatments were added. Medium was changed after 48 hours. SH-11037 synthesized as previously described (Basavarajappa et al. 2015) and dissolved in DMSO was tested at 0.03, 0.3, and 1 µM concentrations for 48 hours. The final concentration of DMSO in each well was 0.2%. Images were taken using an EVOS-fl digital microscope (AMG, Mill Creek, WA, USA) and data were analysed as the sprouting distance in four different directions using ImageJ software v. 1.48v (http://imagej.nih.gov/ij/).

C. In Vivo Experiments

C.1. Intravitreal Injections

Injections were given under anesthesia in a 0.5 µL volume using a 33-gauge needle. The needle was kept in place for 1 min to prevent the reflux of solution when the needle was removed. SH-11037 and t-AUCB were dissolved in DMSO then diluted in PBS to a final concentration of 0.5% DMSO. Vehicle alone (PBS + 0.5% DMSO) was used as control. Eyes were numbed with tetracaine solution before the injection, and triple antibiotic ointment was used immediately after the injection to prevent infection. I was masked during imaging and analysis to avoid bias.

C.2. In Vivo Toxicity

Toxicity of intravitreally injected SH-11037 was initially assessed by histology, immunofluorescence, and retinal function. The presence of acute
ocular toxicity was investigated by injecting SH-11037 at different concentrations (0.1, 1, 10, and 100 µM), while longer-term effects were tested by injecting 10 µM SH-11037. Mice were sacrificed 3 and 14 days post injections, respectively. Eyes were enucleated and fixed in 4% PFA overnight, and then the eyes were paraffin embedded and sectioned at 5 µm thickness by the Indiana University School of Medicine Histology Core. Mayer’s hematoxylin and eosin (H&E) staining was performed (Wenzel et al. 2015), and retinal morphology quantified by calculating the ratio of A (the distance from the ganglion cell layer to the outer edge of the inner nuclear layer) to B (the distance from the ganglion cell layer to the outer edge of the outer nuclear layer) as previously described (Park et al. 2014).

C.3. Immunohistochemistry

Sections were deparaffinised with xylenes and ethanol series and boiled in citrate buffer for 5 minutes. Sections were blocked for one hour with 10% DAKO blocking buffer (DAKO, Carpinteria, CA) in Tris-buffered saline (TBS), then probed overnight at 4°C with primary antibodies, GFAP, cleaved caspase 3, MCP1 and sEH (H-215), diluted 1:400 in 10% DAKO diluent in TBS. Sections were then incubated with Alexafluor 555-conjugated goat anti-rabbit antibody (Life Technologies, Waltham, MA) for 1 hour, 1:400 in TBS. Sections were washed with TBS, dehydrated through an ethanol series, then mounted with Vectashield mounting medium with DAPI (Vector Labs, Burlingame, CA). Images were taken with an LSM700 confocal microscope (Zeiss, Thornwood, NY) with a 40x objective.
C.4. Retinal Electrophysiology and Vasculature Staining

Electroretinograms (ERG) were obtained from animals 14 days post intravitreal injection of 100 µM SH-11037, SH-11037/anti-VEGF164 combination or vehicle control and performed as previously described (Cai et al. 2012). Briefly, scotopic rod recordings were performed on overnight dark-adapted mice, with 10 increasing light intensities of white light and responses were recorded with a visual electrodiagnostic system (UTAS-E 2000; LKC Technologies, Gaithersburg, MD). Stimuli were presented at intensities of 0.025, 0.25, and 2.5 log cd·s/m² at 10-, 20-, and 30-second intervals, respectively. Ten responses were recorded and averaged at each light intensity. Photopic cone recordings were undertaken after mice were light adapted to a white background light of 100 cd·s/m² for 8 min. Recordings were performed with four increasing flash intensities from 0, 5, 10 and 25 log cd·s/m² in the presence of a constant 100 mcd·s/m² rod suppressing background light. The b-wave amplitude was determined from a-wave trough to b-wave peak, behind the last prominent oscillatory potential. Preparation of retina flatmounts and staining of pre-existing vasculature was performed as previously described (Basavarajappa et al. 2015). The retinal vessel area from the superficial plexus was measured from four random fields from four different sections per eye using Adobe Photoshop (CC 2015, v1.2) software and data are presented as the retinal vessel area per unit area of retina analyzed.
C.5. Laser-induced Choroidal Neovascularization

Before laser photocoagulation, eyes were dilated using 1% tropicamide, then underwent laser treatment using 50 μm spot size, 50 ms duration, and 250 mW pulses of an ophthalmic argon green laser, wavelength 532 nm, coupled to a slit lamp. A coverslip was used to allow viewing of the posterior pole of the eye. Each eye received 3 laser burns centered around the optic nerve at 12, 3, and 9 o’clock positions. The appearance of a bubble at the site of laser application was used to identify laser-induced damage of Bruch’s membrane. Lesions in which bubbles were not observed were excluded from the study. The effect of SH-11037 on the L-CNV model using final estimated vitreal concentrations of 0.1, 0.3, 1, or 10 μM SH-11037 was tested. Anti-mouse VEGF164 neutralizing antibody (R&D Systems, Minneapolis, MN) was tested at 0.2, 1, and 5 ng/eye. t-AUCB was tested at 0.1, 1, or 10 μM final estimated concentrations. Vehicle (PBS + 0.5% DMSO) was used as a negative control. All injections were given a single time, immediately or 5 days post-laser treatment as indicated.

C.6. Optical Coherence Tomography and Fluorescein Angiography

OCT was performed at the indicated times using the Micron III intraocular imaging system (Phoenix Research Labs, Pleasanton, CA) or the InVivoVue OCT system (Bioptigen Inc., Research Triangle Park, Durham, NC). These are two of the most commonly used rodent OCT systems. Before the procedure, eyes were dilated with 1% tropicamide solution and lubricated with hypromellose ophthalmic demulcent solution (Gonak) (Akorn, Lake Forest, IL). Mice were then
placed on a custom heated stage that moves freely to position the mouse eye for imaging. Several horizontal and vertical images were taken per lesion to allow calculation of CNV vascular volume.

For the InVivoVue system, experiments were done separately by Michael Boulton's laboratory at IUSM. Pupils of mouse eyes were dilated with 1% atropine and 2.5% phenylephrine hydrochloride. Mice were then anesthetized. One drop of 2.5% hydroxypropyl methylcellulose was administrated to eyes before examination. Three lateral images (nasal to temporal) were collected, starting at the meridian crossing through the center of the optic nerve head (ONH), and the corresponding L-CNV injury spots were identified by contrasts. Composite fundus images (1.4x1.4 mm, 400x100x1x1) were taken centered on the ONH. Corresponding high resolution B-scan (1.4x1.4 mm, 400x400x1x1) images were then obtained from swept L-CNV spots.

Fluorescein angiography (FA) was performed 14 days post laser by intraperitoneal injection of 50 µL of 25% fluorescein sodium (Fisher Scientific, Pittsburgh, PA). Fundus images were taken using the Micron III system and Streampix software.

C.7. Quantification of Lesions as Ellipsoids

Ellipsoid quantification for OCT images were done as described in chapter III, section B.1. Briefly, for images obtained from the Micron system, the widest section of the lesions from perpendicular planes were used to calculate ellipsoid
volume using the formula \( V = \frac{4}{3} \pi abc \) where \( a, b, \) and \( c \) are the radii of the three axes of the ellipsoid using ImageJ software. The volumes of the three lesions in each eye were averaged and considered as an \( n=1 \) for statistical analysis.

C.8. Choroidal Flatmount and Analysis

Fourteen days post-laser, eyes were enucleated and fixed in 4% paraformaldehyde (PFA) overnight. The anterior segment and the retina were removed, and the remaining RPE/choroid/sclera was permeabilized by incubation with 0.3% Triton X-100 followed by incubation with rhodamine labeled *Ricinus communis* agglutinin I (Vector Labs, Burlingame, CA), in the dark for 45 minutes, to stain blood vessels. The staining step was followed by two washes with Tris buffered saline with 0.1% Tween-20 (TBST). Flatmounts of the choroid were prepared and mounted with Vectashield mounting medium (Vector Labs, Burlingame, CA) and Z-stack images were taken on an LSM700 confocal microscope (Zeiss, Thornwood, NY). ImageJ software was used to analyze Z-stack images; the summation of the whole stained area in each section, multiplied by the distance between sections (3 \( \mu \)m) was used as an index for the CNV vascular volume (Qi et al. 2012). The volumes of the three lesions in each eye were averaged and considered as an \( n=1 \) for statistical analysis. This experiment was performed following the guidelines and exclusion criteria described previously (Poor et al. 2014) to ensure reproducibility and eliminate investigator bias. Briefly, a lesion was excluded if (1) it was associated with choroidal hemorrhage; (2) the lesion was not circular in shape; (3) two lesions
fused together; (4) the lesion size was an outlier (too big, or too small) from the other 2 lesions in the same eye; (5) if two lesions were excluded from an eye, the third one was excluded as well.

C.9. Lipid Profiling

C57BL/6J mice underwent laser treatment followed by intravitreal injections of 10 µM SH-11037, 10 µM t-AUCB or vehicle control. Mice were sacrificed 3 days post-laser, eyes were enucleated and retina/choroid layers were immediately separated and stored at -80°C. Lipid profile analysis was performed by the Lipidomics Core Facility at Wayne State University using standard operating procedures developed by the core as previously described (Maddipati and Zhou 2011). The samples were then extracted for PUFA metabolites using C18 extraction columns and subjected to LC-MS analysis as described earlier (Maddipati et al. 2014). Chromatography was performed on a Prominence XR system (Shimadzu) using Luna C18 column. The data were collected using Analyst 1.6.2 software and the MRM transition chromatograms were quantitated by MultiQuant software (both from ABSCIEX).

D. Statistical Analyses

Statistical analyses were performed with GraphPad Prism 6 software. Student’s t-tests were used to compare retinal thickness measurements and ERG parameters. Repeated measures two-way ANOVA with Dunnett’s post hoc test was used for choroidal sprouting distance measurements. The Spearman
correlation coefficient was used to assess the correlation between lesion volumes measured by OCT and choroidal flatmounts. For all other experiments, one-way ANOVA was used with Tukey's post hoc tests to compare between treatments. Two-sided $P$ values $< 0.05$ were considered statistically significant.
CHAPTER III. DEVELOPMENT OF A NOVEL TOOL FOR THE QUANTIFICATION OF CNV LESIONS

(The majority of this chapter was previously published as the following paper: Sulaiman RS, Quigley J, Qi X, O’Hare MN, Grant MB, Boulton ME, Corson TW. A simple optical coherence tomography quantification method for choroidal neovascularization. Journal of Ocular Pharmacology and Therapeutics, 2015, 31 (8), 447-454 – reproduced with permission from MaryAnn Liebert publisher)
A. Chapter Summary

Therapeutic efficacy is routinely assessed by measurement of lesion size using flatmount retinas and confocal microscopy in the laser-induced choroidal neovascularization (L-CNV) rodent model. I investigated whether optical coherence tomography (OCT) quantification, using an ellipsoid volume measurement, was comparable to standard ex vivo evaluation methods for this model and whether this approach could be used to monitor treatment-related lesion changes. Bruch’s membrane was ruptured by argon laser in the dilated eyes of C57BL/6J mice, followed by intravitreal injections of anti-VEGF$_{164}$ or vehicle, or no injection. In vivo OCT images were acquired using Micron III or InVivoVue systems at 7, 10, and/or 14 days post-laser and neovascular lesion volume was calculated as an ellipsoid. Subsequently, lesion vascular volume was compared to that calculated from confocal Z-stack images of agglutinin stained choroidal flatmounts. Ellipsoid volume measurement of orthogonal 2D OCT images obtained from different imaging systems correlated with ex vivo vascular volumes for L-CNV (Spearman’s $\rho = 0.82$, 0.75, and 0.82 at days 7, 10, and 14, respectively). Ellipsoid volume calculation allowed temporal monitoring and evaluation of CNV lesions in response to anti-VEGF treatment. Ellipsoid volume measurements allow rapid, quantitative use of OCT for assessment of CNV lesions in vivo. This novel method can be used with different OCT imaging systems with sensitivity to distinguish between treatment conditions. It may serve as a useful adjunct to the standard ex vivo confocal quantification, to assess
therapeutic efficacy in preclinical models of CNV, and in models of other ocular diseases.

B. Background and Rationale

Choroidal neovascularization (CNV) is the aberrant growth of new blood vessels originating from the choroid into the subretinal space through a break in Bruch’s membrane (de Jong 2006). The L-CNV murine model has become the “gold standard” in preclinical studies (Grossniklaus et al. 2010), despite limitations of using rodents (Lambert et al. 2013). The resultant CNV resembles aspects of exudative AMD and can easily be performed in rodents, producing robust subretinal vascular lesions within 14 days. This model has been invaluable for the evaluation of the effects of drug therapies on CNV lesion progression (Dobi et al. 1989, Sulaiman et al. 2014). As shown later (Chapters IV and V), the L-CNV model was used throughout this study to evaluate the antiangiogenic potential of different compounds.

Targeting the VEGF pathway, using specific antibodies, antibody fragments, or aptamers, is a standard pharmacotherapeutic approach for wet AMD (Prasad et al. 2010) and has been previously tested in L-CNV experimental models (Saishin et al. 2003, Krzystolik et al. 2002). Several methods for 2-dimensional (2D) evaluation of L-CNV lesions have been used including histological analysis and fluorescein angiography (Giani et al. 2011). Measuring L-CNV lesion vascular volume using stained choroidal flatmounts ex vivo allows
3-dimensional (3D) measurement of lesion size, which is more informative than measuring lesion area alone and has become widely accepted as a quantitative method to indicate drug efficacy in ameliorating CNV lesions (Lambert et al. 2013, Sengupta et al. 2003, Sengupta et al. 2005).

Although ex vivo lesion measurements of L-CN V are robust and powerful, in vivo imaging analysis of lesions would allow longitudinal studies. Optical coherence tomography (OCT) is a non-invasive, in vivo imaging technique that generates high resolution, cross sectional images of biological systems (Huang et al. 1991). The technique has found extensive use in ophthalmology (Huang et al. 1991, Chen et al. 2005) providing detailed images for both the anterior and posterior segments of the eye (Drexler et al. 2001) and has become an essential tool for the clinical evaluation of ocular pathologies such as wet AMD (Jia et al. 2014), retinal tumors (Espinoza et al. 2004, Rootman et al. 2013, Shields et al. 2014), and retinal detachment (Wolfensberger and Gonvers 2002).

Paralleling its rise to clinical prominence, OCT has been used preclinically to monitor disease progression, and OCT images have been shown to be comparable to histological characteristics of normal retina (Figure 7) and in disease models (Fischer et al. 2009, Huber et al. 2009, Corson et al. 2014, Wenzel et al. 2015), including L-CN V (Giani et al. 2011, Fukuchi et al. 2001).
Figure 7. OCT is comparable to histology. Representative images of retina from H&E staining (left) and OCT (right) showing different retinal layers. Abbreviations: ganglion cell layer (GCL), inner plexiform layer (IPL), inner nuclear layer (INL), outer plexiform layer (OPL), outer nuclear layer (ONL), inner segment of photoreceptors (IS), outer segment of photoreceptors (OS), retinal pigment epithelium (RPE).

However, 3D quantification of lesions in OCT images has previously used specialized software that is not readily available or compatible with different hardware systems. Moreover, the correlation between calculated lesion vascular volume from OCT images and ex vivo choroidal flatmount 3D quantification has not been shown previously. In this chapter, I show that simple quantification of vascular volumes from OCT images provides reproducible and comparable evaluation to the ex vivo analytical methods used in the L-CNV mouse model.
This ellipsoid quantification method will be used in later chapters for the evaluation of the antiangiogenic potential of compounds on the L-CNV model.

C. Results

C.1. Quantification of Lesions as Ellipsoids

OCT has been extensively used for the preclinical monitoring of disease progression in different models such as the L-CNV model. In order to develop a simple yet reproducible method for the evaluation of CNV lesions in vivo, I investigated whether quantifying the CNV lesion as an ellipsoid would be feasible. The Micron III and InVivoVue are two of the most commonly used OCT imaging systems in preclinical studies, and therefore were used in this study to evaluate the ellipsoid quantification approach. Quantification was performed as in Figure 8, for images obtained from the Micron system. The widest section of the lesions from perpendicular planes were used to calculate ellipsoid volume using the formula $V = \frac{4}{3} \pi abc$ where $a$, $b$, and $c$ are the radii of the three axes of the ellipsoid. The radii were manually drawn and measured using ImageJ software. The CNV lesion borders were defined from the onset of thickening of the choroidal layer horizontally to the borders of the hyperreflective lesion vertically. The lesion borders were defined similarly for images obtained using the InVivoVue system. In this case, quantitative measures, width ($a$) and depth ($b$) of the lesion were measured from the exported central image in B-scanned compositions. The length ($c$) of the lesions was calculated from en face images as the difference in distance between the beginning and the end of the lesion.
Images from both the Micron III and InVivoVue systems were analyzed by two independent graders for interobserver comparison. The volumes of the three lesions in each eye were averaged and considered as an n=1 for statistical analysis.

Figure 8. Determination of ellipsoid axes in Micron OCT images. A. Representative images showing the horizontal plane of OCT imaging (red line) in the bright field fundus photographs and the radii $a$ (width) & $b$ (depth relative to the retina) on the corresponding zoomed in OCT scan. B. Representative images showing the vertical plane in the bright field image and radius $c$ (length) on the corresponding orthogonal zoomed in OCT scan. C. A schematic diagram showing an ellipsoid with labeled axes corresponding to the OCT images, and the equation used in calculating ellipsoid volume. Scale bars = 100 µm.
Abbreviations; ganglion cell layer (GCL), outer nuclear layer (ONL), retinal pigment epithelium (RPE).

C.2. Ellipsoid Quantification In Vivo is Comparable to Ex Vivo Analysis in L-CNV

Although Z-stack analysis of wholemounts gives robust and reproducible data, I sought a simple means of evaluating L-CNV lesion volume in vivo. Mice underwent OCT 14 days post-laser. Comparing volumetric measurements of OCT and agglutinin-stained wholemount Z-stacks, significant correlation was observed by two independent graders; grader 1 with $\rho = 0.79$, $P<0.01$ and grader 2 $\rho = 0.78$, $P<0.05$ (Figure 9). The intraobserver correlation (based on repeated analysis of the same dataset) was $\rho = 0.9$, $P<0.001$, while the interobserver correlation was $\rho = 0.73$, $P<0.05$.

Figure 9. L-CNV lesion vascular volumes calculated from Micron OCT images obtained 14 days post-laser, analyzed by two independent graders, correlate with
those calculated based on choroidal flatmount Z-stacks. Grader 1 (left)
Spearman’s ρ = 0.79, P<0.01. Grader 2 (right) ρ = 0.78, P<0.05. n = 10.

C.3. Ellipsoid Quantification is Sensitive to Different Treatment Conditions

In the L-CNV model, anti-VEGF antibodies have previously been shown to reduce CNV vascular volume, based on Z-stack image quantification, compared to vehicle treated controls (Campa et al. 2008). I first sought to reproduce this amelioration in the CNV lesion size using the L-CNV mouse model and to compare the difference between treatment groups using both confocal microscopy and OCT. Quantification of CNV lesion vascular volume using the standard Z-stack image method showed the expected significant reduction, about 35% (Liu, Qi, et al. 2013), in lesion volume after anti-VEGF treatment compared to vehicle controls (Figure 10).
Figure 10. Choroidal flatmounts stained with agglutinin show a significant reduction in CNV vascular volume after anti-VEGF treatment. A. Representative images from confocal microscopy for CNV lesions from vehicle-treated (*left*) and anti-VEGF-treated eyes (*right*). Scale bars = 50 µm. B. Quantification of the CNV lesion vascular volume from Z-stack images using ImageJ software demonstrated a significant reduction in CNV lesion vascular volume after anti-VEGF intravitreal injection compared to vehicle treated controls. Mean ± SEM, n = 6. Mann-Whitney test, **P<0.01.

Interestingly, CNV lesion volume calculated using this method from OCT images obtained 7 days after laser and injection also demonstrated a significant
reduction, likewise about 35%, after anti-VEGF treatment (Figure 11). Similar results were obtained from images after 14 days (Figure 11).

![Figure 11. Quantification of L-CNV lesions by OCT shows a significant reduction in lesion volume after anti-VEGF treatment. A. Representative images of L-CNV lesions, shown between red brackets, from vehicle-treated (left) and anti-VEGF-treated eyes (right) obtained after 7 days (top) and 14 days (bottom). B. Quantification of the CNV lesion volume calculated as an ellipsoid, showing a significant reduction in CNV lesion volume after anti-VEGF intravitreal injection compared to vehicle treated controls, after both 7 and 14 days. Note different y-axis scales. Mean ± SEM, n = 6. Mann-Whitney test, **P<0.01.](image)

Importantly, OCT volume measurements significantly correlated with those measured by Z-stack quantification, after 7 days (ρ = 0.82, P<0.01) (Figure 12A), and after 14 days (ρ = 0.82, P<0.01) (Figure 12B).
Figure 12. L-CNV lesion volumes following treatments calculated from Micron OCT images correlate with those calculated based on choroidal flatmount Z-stacks. A. OCT calculations at 7 days, Spearman’s $\rho = 0.82$. B. OCT calculations at 14 days, $\rho = 0.82$. Vehicle-treated, blue, and anti-VEGF164-treated eyes, red. $P<0.01$, $n = 6$ per treatment. Note different y-axis scales.

C.4. Ellipsoid Quantification is OCT Platform-Independent

To validate that our ellipsoidal volume measurement technique could be applied to images from another automated OCT system, I evaluated L-CNV lesions using OCT images obtained with the InVivoVue system. Laser burns were applied to mouse eyes and after 10 days, lesions were evaluated by OCT in vivo. Lesion volumes were calculated by ellipsoid volume equation, and again compared to volumetric measurements of CNV vascular volumes from agglutinin stained choroidal flatmounts ex vivo after 14 days. Interestingly, a significant correlation was again observed between ellipsoid volume calculation and confocal Z-stack images (Figure 13) ($\rho = 0.75$, $P<0.05$). Similar correlation values were obtained by a second grader to account for interobserver variability.
Moreover, removing the peak point, which might be an outlier, did not change the correlation ($\rho = 0.75, P<0.05$).

Figure 13. L-CNV lesion volumes calculated from InVivoVue OCT images obtained 10 days post-laser correlate with those calculated based on choroidal flatmount Z-stacks. A. Representative images showing the horizontal plane of OCT imaging (green line) in the en face image and the corresponding B-scan focus on the optic nerve head (ONH). Scale bar = 34 µm. B. Representative images showing the en face image and the corresponding B-scan at an L-CNV lesion (arrow). C. A significant correlation is observed between 3D measurements of OCT images and confocal Z-stack images, Spearman’s $\rho = 0.75, P<0.05$, $n = 10$. This experiment was performed by Judith Quigley and Xiaoping Qi from Michael Boulton’s laboratory.
D. Discussion

OCT imaging represents an essential adjunct in clinical diagnosis and monitoring of numerous ocular diseases, such as AMD (Keane et al. 2012, Rootman et al. 2013). Evaluation of L-CNV in vivo using OCT imaging has been previously studied in different species such as monkeys (Wang et al. 2015, Onami et al. 2012), rabbits (Koinzer et al. 2013), and rats (Liu, Hui, et al. 2013, Jiao et al. 2013, Fukuchi et al. 2001). Others have evaluated OCT in the mouse model of L-CNV used here by comparing OCT imaging to immunostaining and histological analyses (Giani et al. 2011, Berger et al. 2014, Hoerster et al. 2012). However, in this study, I compared for the first time OCT quantification of L-CNV lesions to the robust and commonly used ex vivo agglutinin stained choroidal wholemount quantification method, which allows volumetric measurements of L-CNV lesions through Z-stack sections (Lambert et al. 2013, Sengupta et al. 2003). I saw excellent correlation between the two methods using different OCT imaging systems.

Quantification of OCT images usually requires specialized, proprietary software that is not necessarily compatible with multiple imaging systems. In this study, I showed that quantitative OCT analysis by a simple method allowed in vivo evaluation and monitoring of choroidal lesions in the L-CNV model using two of the most commonly used preclinical OCT imaging systems. Although I used ImageJ for analysis, the radii measurements needed for ellipsoid volume could be done using any image-editing program. Quantification of L-CNV lesions,
obtained from either Micron or InVivoVue OCT images, using an ellipsoid volume calculation, provides reproducible and comparable results to the standard ex vivo quantification method. Consistently, I observed a significant correlation, by two independent graders, between OCT images obtained at day 7, 10, or 14 post-laser, and Z-stack confocal measurements at day 14. However, due to the difference in scaling of the Micron III and InVivoVue imaging systems, calculated lesion volumes by Micron OCT were uniformly larger than those calculated by confocal microscopy. Tissue shrinkage during fixation may also contribute to this finding. Alternatively, since the length of the lesion (dimension c) on the InVivoVue was obtained from en face images directly, not from vertical scans as was done with the Micron III system, this might induce some differences in the final calculation. Thus, using the same volumetric measurement system throughout the analysis of an experiment is important.

Our method also applies to OCT imaging to assess pharmacotherapeutic efficacy. Here, as proof of concept, I used the standard anti-VEGF antibody treatment, versus vehicle treatment, to ameliorate CNV vascular volume. To my knowledge, this is the first use of OCT to show therapeutic effect in the murine L-CNV model, although OCT has been used similarly in macaques (Onami et al. 2012). As I showed, OCT can be used to monitor drug response longitudinally in the same animal, reducing animal usage and enabling time-course comparisons. For instance, I noted that mean L-CNV lesion volumes decreased from Day 7 to
Day 14 (Figure 11, 12), but the distinction between anti-VEGF and vehicle treatments remained about 35% as previously shown (Liu, Qi, et al. 2013).

OCT analysis is also rapid. Preparation of choroidal flatmounts for the quantification of L-CNV vascular volume is a technically involved and time-consuming technique, requiring at least one overnight step and considerable confocal imaging time, as well as expensive reagents (fluorescent agglutinin in particular) (Lambert et al. 2013). Conversely, acquiring multiple images on an OCT system takes about 5–10 minutes per eye followed by 5 minutes for calculation of the lesion volume. Therefore, our ellipsoid volume quantification method represents an inexpensive, rapid, yet reliable quantification method for lesions from OCT images. Despite that, it is important to mention that OCT quantification is not a replacement for the Z-stack confocal measurement. However, it might provide an early prediction of therapeutic effects as well as a second evaluation method for CNV lesion vascular volume.

One limitation of this method is the manual detection of CNV lesion dimensions, which might introduce bias to treatment conditions. However, this can be avoided when the experimenter is masked to treatments throughout imaging and analysis, as well as using at least two independent graders for volumetric analysis. True CNV lesion shape is likely not a simple ellipsoid; therefore, using a simple ellipsoid volume measurement may overestimate irregularly shaped lesions (Hoerster et al. 2012). However, following from this, in
experiments comparing CNV-lesion reducing therapies to control conditions it is likely that our method would be quite conservative (by potentially overestimating lesion volume and thus underestimating treatment effects), which might be considered as a strength. Like any OCT analysis, our method also requires a clear cornea and lens, and lesions in or near the central retina. This method is also limited to disease models that involve localized lesions that can be demarcated by OCT, such as L-CNV. However, a strength of our method is that it can be used to calculate volumes from the L-CNV murine model using different automated imaging systems, specifically the Micron and InVivoVue products. This study provides evidence that OCT is a valuable tool to rapidly and quantitatively evaluate response to drug therapies for choroidal neovascularization using the L-CNV mouse model. Our simple ellipsoidal quantification method may also be applied to the analysis of other focal intraocular lesions and provides rapid analysis for preclinical testing of possible therapeutic agents. Therefore, the novel ellipsoid quantification method could be used in conjunction with the ex vivo confocal quantification for the assessment of L-CNV lesions to evaluate the therapeutic potential of pharmacotherapies such as SH-11037 as will be further discussed in Chapter IV.
CHAPTER IV. EVALUATION OF THE ANTIANGIOGENIC ACTIVITY OF SH-11037 IN CNV-RELEVANT SYSTEMS

(The majority of this chapter forms the following research paper: Sulaiman RS, Merrigan S., Quigley J, Qi X, Lee B, Boulton ME, Kennedy B, Seo SY, Corson TW. A novel small molecule ameliorates ocular neovascularisation and synergises with anti-VEGF therapy. 2016, Scientific Reports. 6, 25509 – reproduced with permission from Nature Publishing Group. Figures 16–19 were published in (Basavarajappa et al. 2015) – reproduced with permission from the American Chemical Society)
A. Chapter Summary

Ocular neovascularization underlies blinding eye diseases such as retinopathy of prematurity, proliferative diabetic retinopathy, and wet age-related macular degeneration. These diseases cause irreversible vision loss, and provide a significant health and economic burden. Biologics targeting vascular endothelial growth factor (VEGF) are the major approach for treatment. However, up to 30% of patients are non-responsive to these drugs and they are associated with ocular and systemic side effects. Therefore, there is a need for small molecule ocular angiogenesis inhibitors to complement existing therapies. I examined the safety and therapeutic potential of SH-11037, a synthetic derivative of the antiangiogenic homoisoflavonoid cremastranone, in models of ocular neovascularization. SH-11037 dose-dependently suppressed angiogenesis in the choroidal sprouting assay ex vivo and in human retinal endothelial cells in vitro. Additionally, intravitreal SH-11037 (1 µM) significantly reduced choroidal neovasculariation (CNV) lesion volume in the laser-induced CNV mouse model, comparable to an anti-VEGF antibody. Moreover, SH-11037 synergized with anti-VEGF treatments in vitro and in vivo. Up to 100 µM SH-11037 was not associated with signs of ocular toxicity and did not interfere with retinal function or pre-existing retinal vasculature. SH-11037 is thus a safe and effective treatment for murine ocular neovascularization, worthy of further mechanistic and pharmacokinetic evaluation.
B. Background and Rationale

Biologics targeting VEGF are the standard treatment for blinding eye diseases; they successfully slow or even reverse vision loss in about 70% of wet AMD patients (Prasad et al. 2010), and are now being used for ROP and PDR. However, repeated intravitreal injections of anti-VEGF drugs can be associated with ocular and systemic side effects such as ocular hemorrhage and stroke (Falavarjani and Nguyen 2013, Shima et al. 2008, Stewart 2012b). Moreover, about 30% of wet AMD patients are nonresponsive to anti-VEGF treatments (Lux et al. 2007). Therefore, there is an unmet need to develop new antiangiogenic molecules to complement and combine with these existing approaches. Such novel therapies might also be useful for other neovascular diseases, including cancers.

Our laboratory (Basavarajappa et al. 2015) and others (Kim, Kim, Kim, et al. 2007, Shim et al. 2004, Kim, Kim, Yu, Jun, et al. 2008) have previously tested the natural homoisoflavonoid, cremastranone, for its antiangiogenic potential in vitro and in vivo in ocular disease models. We recently developed a synthetic derivative of cremastranone, named SH-11037 (Figure 6), using a cell-based structure-activity relationship analysis (Basavarajappa et al. 2015). SH-11037 was more potent than the parent compound, cremastranone, with about 10-fold antiproliferative selectivity towards HRECs over macrovascular endothelial cells, HUVECs and aortic ECs, and had negligible effects on other ocular cell types (Figure 14).
Figure 14. SH-11037 is more potent than the parent compound cremastranone in inhibiting HREC proliferation as measured by alamarBlue proliferation assay. A. A comparison of the GI\textsubscript{50} values of cremastranone (Crem) and SH-11037 on different cell types. B. A dose-response curve of the anti-proliferative activity of SH-11037 on HRECs, a disease-relevant microvascular endothelial cell type. Mean ± SEM, n=3.

A detailed evaluation of the antiangiogenic therapeutic potential and safety of SH-11037 for ocular neovascularization has not been performed previously. In this chapter, I explore SH-11037’s pharmacological activity using endothelial cell proliferation, migration, and tube formation assays in vitro, a choroidal angiogenesis model ex vivo, and in vivo in the L-CNV mouse model as a single treatment immediately after and 5 days post-laser, a stage at which near-maximal CNV lesion size is already reached. I also assessed intraocular toxicity of this compound in mice. I show that SH-11037 has a strong antiangiogenic potential on CNV in the absence of ocular toxic effects, which could make it a
new additive or alternative therapy to existing anti-VEGF drugs for treatment of neovascular diseases in the eye and other tissues.

C. Results

C.1. Validation of SH-11037’s Antiangiogenic Activity In Vitro.

Given the intriguing potency and selectivity of SH-11037 in alamarBlue proliferation assays compared to the parent compound, cremastranone (Figure 14), I tested SH-11037 in a secondary cell proliferation assay, which monitors the incorporation of 5-ethynyl-2′-deoxyuridine, an analogue of thymidine, into the de novo DNA synthesis of proliferating HRECs. Here, I confirmed the dose-dependent inhibition of HREC proliferation by SH-11037 (Figure 15).

Figure 15. SH-11037 inhibits proliferation of HRECs as measured by EdU incorporation assay. A. The nuclei of all cells are labeled with DAPI (blue) and actively dividing cells are labeled with EdU (pink). B. Quantification of percentage

![Figure 15](image-url)
of EdU incorporated cells. Mean ± SEM, n = 3, representative results from at least triplicate experiments. ***P<0.001. Scale bars = 200 µm.

Due to the potency of SH-11037 in inhibiting the proliferation of HRECs, I tested whether these effects are due to induction of apoptosis. I employed activated caspase (Figure 16) and TUNEL (Figure 17) assays to monitor the apoptosis of HRECs in the presence of different concentrations of SH-11037. Less than 10% of HRECs underwent apoptosis when treated with up to 2 µM SH-11037 (Figure 17), indicating that the compound may not be cytotoxic at effective concentrations.

Figure 16. SH-11037 caused negligible apoptosis as assayed by activated caspase 3 immunofluorescence. A. Representative images of HRECs where the nuclei are labeled with DAPI (blue) and activated caspase 3 is shown in pink, 1 µM staurosporine (SP) is positive control. B. Quantification of percentage of
apoptotic cells. Mean ± SEM, n = 3, representative results from at least triplicate experiments. Scale bars = 200 µm. (Basavarajappa et al. 2015)

Figure 17. SH-11037 does not cause a significant induction of apoptosis as evaluated by TUNEL assay. A. Representative images of HRECs where the nuclei are labeled with DAPI (blue) and TUNEL+ cells undergoing apoptosis are shown red. B. Quantification of percentage of apoptotic cells. Mean ± SEM, n = 3, representative results from at least triplicate experiments. Scale bars = 200 µm. (Basavarajappa et al. 2015)

After establishing the anti-proliferative activity of this promising lead, I further tested its effect on different antiangiogenic properties in vitro. I monitored the migration of HRECs, an important activity of endothelial cells during new blood vessel formation in the presence of SH-11037 in a scratch wound assay (Figure 18).
Figure 18. SH-11037 dose-dependently suppresses endothelial cell migration as demonstrated by scratch wound assay. A. Representative images of HRECs after 8 hours treatment with different concentrations of SH-11037. B. Quantification of the numbers of migrated cells as a percentage of the DMSO control. Mean ± SEM, n = 3, representative results from at least triplicate experiments. **P<0.01, ***P<0.001. Scale bars = 200 µm. (Basavarajappa et al. 2015)

Matrigel tube formation assay is one of the important in vitro assays, which recapitulates most of the events of physiological angiogenesis such as migration, proliferation, and cell-cell adhesion. I tested the ability of HRECs to form tube-like structures in the presence of increasing concentrations of SH-11037. Interestingly, SH-11037 inhibited the tube formation ability of HRECs in the Matrigel assay at sub-micromolar concentrations (Figure 19).
Figure 19. SH-11037 inhibits tube formation ability of HRECs in vitro. A. Representative images of HRECs plated on Matrigel 8 hours after treatment with different SH-11037 concentrations compared to the DMSO control. B. Quantification of the number of polygons formed as a percentage of the DMSO control. Mean ± SEM, n=3, representative results from at least triplicate experiments. *P<0.05, **P<0.01, Scale bars = 200 µm. (Basavarajappa et al. 2015)

C.2. SH-11037 Inhibits Choroidal Neovascularization Ex Vivo in The Choroidal Sprouting Assay

To investigate the effect of SH-11037 on choroidal angiogenesis, I first tested different concentrations of SH-11037 on the sprouting of mouse choroidal tissues ex vivo. After 48 hours of incubation with SH-11037, the ability of the choroidal tissues to form vascular sprouts of endothelial cells and their microenvironment was significantly inhibited in a concentration-dependent
manner compared to the vehicle treated controls (Figure 20A, B). Sprouting was absent at the highest concentrations tested. This effect was due to suppression of endothelial cell sprouting ability as demonstrated by isolectin B4 staining for endothelial cells in 1 µM SH-11037-treated choroidal tissues compared to vehicle-treated control (Figure 20C). Trypan blue staining for viable cells indicated the absence of significant cell death in SH-11037 treated wells compared to DMSO controls (Figure 20D); this is consistent with the lack of apoptosis induction by SH-11037 in HRECs (Figure 17).
Figure 20. SH-11037 inhibits choroidal sprouting in a concentration-dependent manner without affecting cell viability. A. Representative images of choroidal sprouts formed 48 hours after treatment with indicated SH-11037 concentrations or DMSO control, Scale bars = 1000 µm. B. Quantification of sprouting distance from the edge of the choroid tissue piece to the end of the sprouts averaged from four perpendicular directions using ImageJ software. C. Representative images of isolectin B4-stained endothelial cells in the choroidal sprouts, isolectin (green),
DAPI (blue), scale bars = 50 µm. D. Representative images of trypan blue stained choroidal sprouts 48 hours after treatment with DMSO and 1 µM SH-11037 to assess cell viability, Scale bars = 1000 µm. Mean ± SEM, n=4 from two independent experiments. Two-way ANOVA, Dunnett’s post hoc test, ***P<0.001.

C.3. Intravitreal Injection of SH-11037 Does Not Cause Acute or Chronic Ocular Toxic Effects.

Before evaluating the therapeutic potential of SH-11037 injections in mouse eyes, I first investigated possible toxicity. I examined signs of acute (3 days post injection) and longer-term (14 days post injection) retinal toxicity of intravitreally injected SH-11037 up to 100 µM final concentration in the eye. Hematoxylin and eosin (H&E) stained retinal sections of SH-11037 treated eyes revealed no histological changes compared to vehicle treatment and no injection control eyes (Figure 21A). Quantification of retinal thickness demonstrated the absence of morphological changes between SH-11037 treatments compared to vehicle-treated and uninjected eyes at 3 and 14 days post injections. To further investigate any toxic effects of SH-11037 beyond morphology, retinal sections were stained for glial fibrillary acidic protein (GFAP), cell death markers (cleaved caspase 3, TUNEL) and monocyte chemotactic protein-1 (MCP1) to examine any signs of retinal injury, apoptosis or inflammation, respectively. Neither short- nor longer-term SH-11037 treated retina showed any significant increases of GFAP, cleaved caspase 3, TUNEL staining and MCP1 compared to vehicle-treated
controls (Figure 21B). These data suggest the absence of toxic effects of SH-11037 on retina examined 3 and 14 days post injections.

Figure 21. Intravitreal injection of SH-11037 does not cause ocular toxicity after 3 and 14 days. A. Representative images of H&E stained retinas from vehicle and SH-11037 treatments, and no injections control, and quantification of the indicated A/B ratio show the absence of short- or longer-term changes in retinal layers at any given concentration of SH-11037. Mean ± SEM, n=8 eyes/treatment. Student’s t-tests, P>0.05. Scale bars = 100 µm. B. Immunofluorescence staining revealed no differences between SH-11037 treatment and vehicle control in GFAP, cleaved caspase 3, TUNEL, and MCP1 (each red) as markers of retinal injury, apoptosis, or inflammation, respectively. Laser injured eyes were stained for the same markers as a positive control; white arrows indicate the increased expression of respective markers. Nuclei are stained with DAPI (blue). Scale bars = 20 µm. GCL, ganglion cell layer; ONL, outer nuclear layer; RPE, retinal pigment epithelium.
C.4. SH-11037 Does Not Affect Retinal Function and Pre-existing Vessels.

Given the effects of SH-11037 on angiogenesis in vitro and ex vivo, I examined whether SH-11037 would cause regression of pre-existing retinal vasculature or damage to retinal function. Whole retina flatmounts were prepared 14 days after 100 µM final SH-11037 intravitreal concentration or vehicle intravitreal injections and stained with isolectin B4 (Figure 22A). No changes in the pre-existing retinal vessels were observed after SH-11037 treatment compared to the vehicle control (Figure 22B). Moreover, electroretinography (ERG) was used to evaluate changes in the function of neural retina 14 days after 100 µM SH-11037 injections. Scotopic a- and b-waves, and photopic b-waves were not significantly different in SH-11037 treated eyes relative to the control eyes (Figure 22C,D). These results demonstrate that SH-11037 does not interfere with the function of neural retina or the maintenance of normal retinal vasculature.
Figure 22. SH-11037 does not interfere with retinal function and pre-existing vasculature. A. Isolectin-stained retinal vasculature does not differ between 100 µM SH-11037 and vehicle treated control eyes 14 days post-injection. Scale bars = 50 µm. B. Quantification of retinal vasculature as vessel area per unit area of retina analyzed shows no difference between SH-11037 and vehicle control treatments. C. Representative mean ERG responses. D. Quantification of scotopic a- and b-waves and photopic b-wave shows no difference in retinal function (stimulus: scotopic = 2.5, photopic = 25 cd·s/m²). Mean ± SEM, n=6 mice/treatment. Student’s t-test, P>0.05.

C.5. SH-11037 Significantly Suppresses CNV Lesion Vascular Volume.

After showing that SH-11037 was non-toxic in the eye, to examine the ability of this compound to reduce choroidal neovascularization in vivo, I used the
standard mouse L-CNV model (Lambert et al. 2013). Intravitreal injections of different SH-11037 final concentrations in the eye (0.1, 0.3, 1, 10 µM) were given to C57BL/6J mice immediately after laser application in the experimental design shown in Figure 23.

Figure 23. Experimental design to evaluate the therapeutic potential of SH-11037 in the L-CNV mouse model in vivo.

The CNV vascular volumes in the SH-11037 treated eyes were significantly lower than those in vehicle treated eyes in a dose-dependent manner at 7 days post-laser as monitored in vivo by OCT and measured by ellipsoid volume quantification (Chapter III) (Figure 24A, D). These decreases were comparable to those induced by an anti-VEGF164 antibody, which is a murine-optimized equivalent of bevacizumab, the standard of care in humans (Irani et al. 2016). Additionally, fluorescein angiography revealed reduced leakiness of CNV lesions from SH-11037 and anti-VEGF164 treated eyes relative
to the vehicle treatment (Figure 24B). Confocal images of agglutinin-stained choroidal flatmounts revealed a reduction in CNV lesion size at 1 and 10 μM SH-11037 and anti-VEGF164 treated eyes compared to vehicle controls (Figure 24C). Although there was no reduction in the CNV vascular volume compared to the vehicle control in eyes treated with SH-11037 at 0.1 and 0.3 μM, there was a dose-dependent reduction of CNV vascular volume of about 42% at 1 μM and 55% at 10 μM SH-11037 compared to the control eyes (P<0.01) (Figure 24E). Interestingly, these antiangiogenic effects were comparable to the anti-VEGF164 treatment that demonstrated about 50% inhibition of CNV lesion vascular volume.
Figure 24. SH-11037 dose-dependently suppresses CNV vascular volume. A. Representative OCT images obtained 7 days post-laser, showing CNV lesions of vehicle (left), anti-VEGF164 (middle), and 1 µM SH-11037 (right). Scale bars = 100 µm. B. Representative images from fluorescein angiography 14 days post-laser. C. Representative images from confocal microscopy for agglutinin-stained
CNV lesions 14 days post-laser, scale bars = 50 µm. D. Quantification of CNV lesion vascular volumes from OCT images at day 7 using ellipsoid volume measurement (Chapter III). E. Quantification of CNV vascular volumes from Z-stack images at day 14 using ImageJ software. Mean ± SEM, n=12 eyes/treatment. One-way ANOVA, Tukey’s post hoc tests, **P<0.01, ns; non significant.

C.6. Treatment with SH-11037 Prevents CNV Lesion Progression

From a clinical perspective, drug treatments are usually given to wet AMD patients after CNV development. Therefore, after demonstrating the dose-dependent inhibition of CNV lesion development by SH-11037 given immediately after laser treatment, I tested the therapeutic potential of delayed SH-11037 treatments on pre-existing CNV lesions in the L-CNV mouse model. Intravitreal injections of 10 µM SH-11037, 5 ng anti-VEGF164 and vehicle control were given 5 days post-laser treatment. Mice were then sacrificed 14 days post-laser to evaluate CNV vascular volumes using agglutinin-stained choroidal flatmounts. Interestingly, SH-11037 significantly suppressed CNV lesion vascular volumes relative to the vehicle treatment. This effect was comparable to the standard anti-VEGF164 antibody indicating a strong therapeutic potential of SH-11037 (Figure 25). In order to understand whether SH-11037 only prevents further development of CNV lesions or resolves pre-existing ones, CNV vascular volumes were evaluated 5 days post-laser before the administration of any treatments. Interestingly, 5 days control eyes were significantly lower than the vehicle
treatment. However, there was no significant difference between the 5 days control and SH-11037 and anti-VEGF164 treatments, suggesting that both treatments worked mainly by blocking further growth of CNV lesions (Figure 25).

Figure 25. Delayed treatment of SH-11037 suppresses CNV lesion progression.

A. Representative images from confocal microscopy of agglutinin stained CNV lesions for 5 ng anti-VEGF164 and 10 μM SH-11037 compared to vehicle treatment and 5 days control, scale bars = 50 μm. B. Quantification of CNV lesion vascular volumes from Z-stack images at day 14 using ImageJ software. Mean ± SEM, n=12 eyes/treatment. One-way ANOVA, Tukey’s post hoc tests, *P<0.05, ***P<0.001.
D. Discussion

Angiogenesis is a highly regulated process that involves the formation of new blood vessels from existing ones, which is promoted by positive (proangiogenic) mediators such as VEGF (Carmeliet and Jain 2011). Uncontrolled angiogenesis has been implicated in various pathological conditions, such as cancer and inflammation, and notably in blindness-causing diseases. Due to the existing limitations of anti-VEGF biologics (Saint-Geniez et al. 2008, van Wijngaarden et al. 2005), and the fact that the pathological formation of blood vessels in the eye involves other angiogenic and inflammatory pathways (Yoo and Kwon 2013), the development of new small molecule inhibitors of ocular angiogenesis is crucial to complement the existing biologic therapies. Currently, there are no FDA approved small molecules for ocular neovascularization. However, several promising small molecules are currently in Phase 1 or 2 clinical trials for the treatment of wet AMD such as squalamine, X-82 and PAN-90806 (www.clinicaltrials.gov) (Pecen and Kaiser 2015).

Interestingly, squalamine, which has been recently approved for a Phase 3 trial, is a natural aminosterol derived from dogfish shark cartilage (Pecen and Kaiser 2015), and underwent several evaluation steps in vitro on endothelial cells (Sills et al. 1998), ex vivo in an angiogenesis assay (Sills et al. 1998), and in vivo in the OIR and L-CNV murine models (Higgins et al. 2000, Ciulla et al. 2003). Small molecules such as squalamine, and our compound, SH-11037, have the advantage of possible optimization for topical administration over high molecular weight biologics that require localized drug delivery through intravitreal injection.
In this chapter, I report the pharmacological activity and safety of SH-11037 in the context of ocular angiogenesis. Endothelial cells from different vascular beds have different physiological properties (Bhasin et al. 2010). Additionally, endothelial cells vary within the same organ. Choroidal and retinal endothelial cells are known to possess different properties as indicated by their preferential susceptibility to certain diseases such as diabetic retinopathy versus wet AMD, respectively (Stewart et al. 2011). However, given that both retinal and choroidal endothelial cells are microvascular in nature, and the limitations in obtaining human choroidal endothelial cells due to limited number of donor eyes and commercial unavailability, I tested the antiangiogenic activities of SH-11037 in vitro in HRECs. SH-11037 demonstrated a potent dose-dependent inhibition of HREC proliferation, migration and tube formation without inducing apoptotic cell death, suggesting a cytostatic rather than a cytotoxic activity of SH-11037. Then, I sought to specifically evaluate the effects of SH-11037 in AMD-relevant microvascular tissues of the choroid ex vivo, taking advantage of the capability of the choroidal sprouting assay to measure microvascular angiogenesis in the choroid (Shao et al. 2013). SH-11037 demonstrated a potent inhibitory concentration, which was consistent with the growth inhibitory concentration observed in vitro in retinal microvascular endothelial cells (Figure 14).

In an initial experiment, SH-11037 suppressed retinal neovascularization in the OIR model of ROP (Basavarajappa et al. 2015). Interestingly, up to 100 µM SH-11037, which is at least 10 fold higher than the doses used for in vivo efficacy
assessment, was not associated with short or longer-term signs of ocular toxicity in mouse retina, when the compound was injected intravitreally. It did not interfere with retinal function or the existing retinal vasculature, suggesting that SH-11037 specifically targets proliferating endothelial cells as shown above. However, although I did not observe any gross systemic effects of intravitreal SH-11037, the systemic toxicology of this compound remains to be evaluated. Additionally, to evaluate the therapeutic potential of SH-11037 in a more clinically relevant approach, delayed administration of SH-11037 was evaluated. Interestingly, SH-11037 intravitreal injections suppressed CNV lesion vascular volume when given 5 days post-laser induction in the L-CNV model. This indicates that SH-11037 blocks further growth of CNV lesions but does not resolve the pre-existing ones. However, this might be consistent with the irreversible damage caused by CNV progression in patients after which they are refractory or unresponsive to anti-VEGF drug treatments (Lux et al. 2007).

I have evaluated SH-11037’s pharmacological activity as an antiangiogenic small molecule in different models, from HRECs in vitro, to an ex vivo system of the choroid, and finishing with a disease-relevant mouse model of CNV. Additionally, I extensively investigated the ocular toxicity of SH-11037 by retinal histology, markers of retinal damage, retinal vasculature and retinal function. SH-11037 might not be a suitable candidate for oral dosing due its limited water solubility. However, as a small molecule, it could potentially be optimized for topical applications, which would be appealing as a non-invasive
local delivery route, while still minimizing systemic side effects. Topical delivery could also enable frequent dosing. Alternatively, delayed-release formulations of this small molecule therapy for intravitreal injection might be possible if necessary, although our finding that a single injection of compound has an effect on L-CNV assessed two weeks later suggests a reasonable half-life.

Determination of the pharmacokinetics of SH-11037 will be an essential step to further improve SH-11037 efficacy and route of administration. Despite the power of anti-VEGF therapies, improvement of drug treatments for wet AMD and other neovascular eye diseases – and neovascularization elsewhere in the body – will become necessary with increasing population age.
CHAPTER V. IDENTIFICATION OF THE MECHANISM OF ACTION OF
SH-11037

(Some of this chapter was previously published in the following research paper:

Sulaiman RS, Merrigan S., Quigley J, Qi X, Lee B, Boulton ME, Kennedy B, Seo SY, Corson TW. A novel small molecule ameliorates ocular neovascularisation and synergises with anti-VEGF therapy. 2016, Scientific Reports. 6, 25509 – reproduced with permission from Nature Publishing Group.)
A. Chapter Summary

The standard-of-care therapeutics currently available for the treatment of ocular neovascular diseases, such as wet AMD, aim at targeting the VEGF signaling pathway using biologics. There are no FDA approved small molecules for the treatment of these diseases. Therefore, there is an unmet need to develop novel antiangiogenic, VEGF-independent small molecule inhibitors of ocular angiogenesis to complement or perhaps combine with existing medications. In the previous chapter, I characterized the safety and therapeutic potential of SH-11037 in models of choroidal neovascularization. While SH-11037 demonstrated a dose-dependent suppression of CNV in vivo in the L-CN model, its mechanism of action remained to be elucidated. In this chapter, I show that SH-11037 synergizes with anti-VEGF treatments in vitro and in vivo, suggesting a VEGF-independent mechanism. An SH-11037 protein partner was identified using a photoaffinity pulldown approach to be soluble epoxide hydrolase (sEH), a key enzyme for the metabolism of ω-3 and ω-6 epoxy fatty acids. SH-11037 inhibits sEH enzymatic activity in vitro and in vivo in the context of L-CN.

Despite the low expression levels of sEH in retinal endothelial cells, sEH levels are dramatically upregulated in retinal sections from the L-CN mouse model compared to control. A specific sEH inhibitor, t-AUCB, significantly suppressed CNV vascular volume in the L-CN mouse model in a dose-dependent manner. SH-11037 is thus a novel inhibitor of sEH that could potentially work as an effective VEGF-independent treatment for choroidal neovascularization.
B. Background and Rationale

The only FDA-approved drugs currently existing for the treatment of wet AMD aim at targeting the same pathophysiological mechanism, the VEGF signaling pathway (Prasad et al. 2010). Moreover, all these drugs are biologics. While these biologics are successful in the treatment of wet AMD, unresponsiveness in many cases and the presence of ocular and systemic side effects from inhibiting such a major angiogenic pathway complicate their use (Falavarjani and Nguyen 2013, Shima et al. 2008, Lux et al. 2007). Therefore, there is a pressing need to identify alternative VEGF-independent angiogenic targets that could form the basis for new therapeutics to complement and combine with the existing medications.

Given the potent antiangiogenic activities of SH-11037 in vitro and in vivo in the L-CNV model (Chaper IV), a clear characterization of its mechanism of action is worthy of investigation. I first evaluated a VEGF-independent mode of action of SH-11037 by testing different combinations of SH-11037/anti-VEGF in vitro and in vivo, and then I used a forward chemical genetics approach to identify its protein targets to uncover novel, potentially druggable angiogenic mediators. This led to the identification of sEH as a potential target for SH-11037.

Soluble epoxide hydrolase (sEH) is a 62 kDa bifunctional enzyme that has N-terminal lipid phosphatase and C-terminal epoxide hydrolase activities (Harris and Hammock 2013). While the physiological role of the lipid phosphatase
activity of sEH is not fully understood, its epoxide hydrolase activity has been extensively studied due to its role in the metabolism of arachidonic acids’ epoxide intermediates, epoxyecosatrienoic acids (EETs) (Panigrahy et al. 2011, Zhang et al. 2014). EETs have been shown to play a role in hypertension, pain and inflammation; therefore, sEH inhibitors are currently in clinical trials for their therapeutic potential in cardiovascular protection (Morisseau and Hammock 2013).

Meanwhile, sEH is also involved in the metabolism of ω-3 fatty acids, docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA) epoxides (Harris and Hammock 2013). Interestingly, with regard to angiogenesis, DHA epoxides have potent antiangiogenic effects through a VEGF-independent pathway (Zhang et al. 2013), in contrast to EETs, which have proangiogenic effects resulting in accelerating tumor growth (Panigrahy et al. 2012). Interestingly, DHA levels are known to be higher than those of arachidonic acids in the retina (Arterburn et al. 2006). Additionally, epoxy metabolites of DHA, epoxydocosapentaenoic acids (EDP) have been shown to significantly ameliorate retinal and choroidal angiogenesis (Hu et al. 2014, Yanai et al. 2014). Therefore, inhibition of sEH using small molecules might produce similar effects by enhancing the levels of EDP, with subsequently increased antiangiogenic and anti-inflammatory activities.
In this chapter, I identified sEH as a protein target of SH-11037 using a photoaffinity pulldown approach. Additionally, I showed that SH-11037 inhibited the enzymatic activity of sEH in vitro and in vivo and that the inhibition of the epoxide hydrolase activity of sEH, using a small molecule inhibitor, suppressed CNV lesion vascular volume in vivo. This clearly demonstrates SH-11037 as a VEGF-independent, ocular selective antiangiogenic small molecule that could be used alone or in combination with anti-VEGF drugs for the treatment of wet AMD.

C. Results

C.1. SH-11037 Cooperates with Anti-VEGF Therapy In Vitro and In Vivo.

Since most of the complications of anti-VEGF injections are concentration or dosing frequency dependent (Fraunfelder 2005, Klein et al. 2009), the possibility of reducing anti-VEGF dosage by combining with small molecules is highly desirable. I first tested whether SH-11037 and aflibercept (also known as VEGF Trap) would have a combined effect on the proliferation of HRECs using an alamarBlue fluorescence assay. Different concentrations of aflibercept (50, 200, 400, and 800 µg/ml) were tested alone and in combination with 0.5 µM SH-11037. Interestingly, HREC proliferation was significantly inhibited in the presence of combined treatments more than each treatment alone (Figure 26A). To investigate the nature of the combined effect produced, excess over highest single agent (HSA) and excess over Bliss additivity were calculated (Chapter II) (Figure 26B). Values greater than zero observed in both analyses indicate synergistic effects of the tested aflibercept concentrations with 0.5 µM SH-11037.
Then, I tested the potential of SH-11037 and anti-VEGF164 combinations in vivo in the L-CNV mouse model. I first established a dose-response effect of intravitreal injections of SH-11037 and anti-VEGF164 separately (Figure 24E, 26D). Based on these results, a combination of the lowest fully active dose of SH-11037, 1 µM, and the suboptimal dose of anti-VEGF164, 1 ng/eye (Figure 26C) was chosen. Analysis of confocal Z-stack images of agglutinin stained choroidal flatmounts revealed a significant reduction in neovascularization with the combination therapy compared to anti-VEGF164 alone, \( P<0.01 \) (Figure 26E). Moreover, a combination of individually inactive doses of SH-11037 (0.3 µM) and anti-VEGF164 (0.2 ng/eye) caused a significant suppression of CNV vascular volume compared to SH-11037 and anti-VEGF164 alone, \( P<0.0001 \), and \( P<0.05 \), respectively (Figure 26E) without interfering with retinal function as examined by ERG (Figure 26F-G). Interestingly, this combination was synergistic, with percent inhibition of 37 and 36 in excess over HSA and Bliss additivity, respectively.
Figure 26. SH-11037 synergizes with anti-VEGF therapy in vitro and in vivo. A.

The effects of 0.5 µM SH-11037, different concentrations of aflibercept, and SH-
105/afibbercept combinations on HREC proliferation were tested using an alamarBlue fluorescence assay, Mean ± SEM, n=3. Representative data from duplicate experiments. One-way ANOVA with Tukey's post hoc tests, ***P<0.001.

B. Calculations of the excess % inhibition over highest single agent (HSA) activity and excess over Bliss additivity (see Chapter II). Values ≤ 0 are colored in grey, values from 1–10 are in blue, values >10, indicating synergy, are yellow squares.

C. Representative images from confocal microscopy of agglutinin stained CNV lesions for combination treatment; 1 ng anti-VEGF/1 µM SH-11037 (Combo (1/1)) compared to individual SH-11037 and anti-VEGF164 treatments and vehicle control, scale bars = 50 µm. D. Dose-dependent inhibition of the volume of CNV lesions by anti-VEGF164 injections. No difference was observed between anti-VEGF164 + vehicle compared to anti-VEGF164 alone. E. Quantification of CNV lesion vascular volume shows a substitution of anti-VEGF164 by SH-11037. The combination of 1 ng anti-VEGF164 with 1 µM SH-11037 produces a similar effect to that observed by the 5 ng dose of anti-VEGF164 alone. The combination of 0.2 ng anti-VEGF164 with 0.3 µM SH-11037 (Combo (0.2/0.3)) is significantly different from individual treatments alone, and exceeds Bliss additivity and HSA by 36 and 37%, respectively, indicating synergy. Graphs in D and E are quantification of CNV lesion vascular volumes from Z-stack images. Mean ± SEM, n=12 eyes/treatment. One-way ANOVA, Tukey's post hoc tests, *P<0.05, **P<0.01, ****P<0.0001, ns; non-significant. F. Representative mean ERG responses of combo (0.2/0.3) and vehicle treatments. G. Quantification of scotopic a- and b- waves and photopic b-wave shows no difference in retinal
function between these treatments (stimulus: scotopic = 2.5, photopic = 25 cd·s/m²). Mean ± SEM, n=6 eyes/treatment. Student’s t-test, \( P>0.05 \).

C.2. SH-11037 Does Not Interfere with VEGFR2 Levels and TNFα Signaling

The synergistic effects of SH-11037 and anti-VEGF combination observed in vitro and in vivo suggests a VEGF-independent mechanism of SH-11037 in inhibiting angiogenesis. In order to confirm this observation, HRECs were treated with different concentrations of SH-11037 in serum-free medium followed by 20 minutes stimulation with VEGF-A to specifically test the VEGF signaling pathway. Activating phosphorylation of VEGFR2 and ERK1/2 was then assessed by immunoblot. As expected, SH-11037 did not interfere with VEGFR2 phosphorylation but inhibited ERK1/2 phosphorylation only at higher concentrations (Figure 27A, C).
Figure 27. SH-11037 does not interfere with VEGFR2 activation and TNFα signaling. A. Immunoblots of HRECs treated with different concentrations of SH-11037 followed by a short incubation with VEGF-A showing no difference in phosphorylation of VEGFR2. B. Immunoblots of HRECs treated with different concentrations of SH-11037 followed by TNFα stimulation demonstrate IκB degradation and phosphorylation of p38. C. Quantification of the ratios of pERK/total ERK after VEGF stimulation (left), pVEGFR2/total VEGFR2 after VEGF stimulation (middle), and pP38/total P38 after TNFα activation (right). Mean ± SEM, n=3. Representative data from duplicate experiments. One-way ANOVA with Dunnet’s post hoc tests, *P<0.05.

The TNFα pathway is an important signaling pathway that plays a role in cell migration, proliferation and inflammation (Munoz-Chapuli et al. 2004). A cremastranone isomer has been shown to inhibit TNFα-induced p38MAPK and NFκB activation and nuclear translocation following TNFα stimulation (Basavarajappa et al. 2014). Therefore, I sought to evaluate whether SH-11037 works by a similar mechanism to its parent compound, cremastranone. Interestingly, SH-11037 did not interfere with TNFα-induced phosphorylation of p38 and IκB degradation indicating activation and nuclear translocation of NFκB (Figure 27B, C). Together these data suggest that SH-11037’s mode of antiangiogenic action is VEGF-independent.
C.3. Soluble Epoxide Hydrolase is a Target of SH-11037

In order to use an unbiased approach to identify the molecular targets of SH-11037, I employed a photoaffinity pulldown technique. Two affinity reagents AR1 and AR2 were synthesized by our collaborator Dr. Seo’s laboratory. The ester group in AR1 (shared with SH-11037) was replaced by an amide linkage in AR2, which might increase the stability of AR2 in the experiment. SH-11037-based affinity reagents, AR1 and AR2 were immobilized on streptavidin beads and used to pull down protein binding partners of SH-11037 from porcine brain lysate (Figure 28A). AR2 but not the negative control reagent pulled down a specific protein target, which was identified using mass spectrometry to be sEH (Figure 28B). Immunoblot of eluates from photoaffinity pull down experiments confirmed the identity of the pulled down protein using an sEH specific antibody (Figure 28C).

Following the identification of sEH as a binding target of SH-11037, I tested whether SH-11037 would interfere with the epoxide hydrolase activity of sEH, using an sEH inhibitor screening assay, compared to t-AUCB as a positive control. Trans-4-(4-[3-adamantan-1-yl-ureido]-cyclohexyloxy)benzoic acid (t-AUCB; 20; Figure 28D) is a specific inhibitor of the epoxide hydrolase activity of sEH. It is the most commonly used sEH inhibitor in preclinical studies due to its high solubility and potency with limited toxicity (Zhang et al. 2007, Fromel et al. 2012). Interestingly, SH-11037 inhibited sEH enzymatic activity in vitro in a concentration-dependent manner (Figure 28D). To test whether these effects are
specific to SH-11037, I used as a negative control SH-11098 (16), which is an analog of SH-11037 that was found to be inactive in angiogenesis assays in vitro (Basavarajappa et al. 2015). Indeed, sEH inhibition was mediated by SH-11037 as demonstrated by the lack of inhibitory activity of SH-11098.
Figure 28. Soluble epoxide hydrolase (sEH) is a target of SH-11037. A. Chemical structures of SH-11037, affinity reagent 1 (AR1), affinity reagent 2 (AR2), and negative control reagent (NC). B. Proteins pulled down with indicated reagents.
were separated by SDS-PAGE and silver stained, then identified by mass spectrometry. A unique band was present in AR2 but not NC, red arrow. C. Immunoblot of pulled down protein using antibody against sEH. D. SH-11037 (15) but not its inactive analog SH-11098 (16) significantly suppressed sEH enzymatic activity in vitro. The specific sEH inhibitor t-AUCB (20) was used as a positive control. Mean ± SEM, one-way ANOVA, Tukey’s post hoc tests, *P<0.05, **P<0.01. Silver-stained gel and immunoblots are representatives from at least two independent experiments.

C.4. sEH is Upregulated in L-CNВ Retinal Sections

sEH is widely expressed in various tissues, with the highest levels of expression seen in liver, kidney, and brain in neuronal cell bodies and astrocytes (Norwood et al. 2010, Sura et al. 2008). Given the potent antiangiogenic effects of SH-11037 on HRECs in vitro, and its binding to and inhibition of sEH enzyme, I tested whether the known sEH inhibitor t-AUCB would have similar antiangiogenic effects on HRECs. Surprisingly, t-AUCB failed to inhibit the tube formation ability of HRECs at any tested concentration and up to 10 µM (Figure 29A). Based on this, it was important to investigate the levels of sEH in HRECs. Immunofluorescence staining (Figure 29B) showed the absence of sEH protein levels in HRECs. However, qRT-PCR detected low levels of EPHX2 (sEH) mRNA in HRECs (Figure 29C), suggesting that sEH is present in HRECs with protein levels below the detection threshold of sEH antibody in immunofluorescence. Intriguingly, staining for sEH in retinal sections from L-CNВ
treated mice demonstrated a substantial upregulation of sEH levels in the inner plexiform, outer plexiform, and photoreceptor layers compared to untreated eyes (Figure 29D). This substantial upregulation of sEH in L-CNV was further confirmed in immunoblots of retina and choroid layers of laser treated mouse eyes relative to untreated controls, suggesting a role for sEH in the L-CNV model (Figure 29E).

Figure 29. sEH is upregulated in L-CNV but not in retinal endothelial cells. A. Representative images of the lack of effect of sEH inhibitor t-AUCB on the tube formation ability of HRECs. Scale bars = 200 µm. B. Representative images of immunofluorescence staining of sEH (red) and DAPI for nuclei (blue) in hepatocellular carcinoma HepG2 cells as a positive control compared to HRECs in which sEH was not detected. Scale bars = 50 µm. C. Quantification of EPHX2 (sEH) mRNA levels in HepG2 and HRECs. Cycle threshold (Ct) values equals
26.9 and 33.5, respectively. D. Representative images of retinal sections from L-CNV and control eyes stained with DAPI (blue), agglutinin (green), and sEH (red, arrowheads), showing upregulation of sEH in L-CNV sections. Scale bars = 20 µm. E. Immunoblot of sEH protein levels in mouse retina and choroid sections of laser treated mouse eyes compared to untreated controls, β-actin is a loading control.

C.5. t-AUCB Suppresses CNV Vascular Volume in a Dose-Dependent Manner

Although sEH was not expressed in retinal endothelial cells, the substantial upregulation of sEH in L-CNV eyes strongly suggested a role for sEH in the CNV process. Accumulation of epoxy metabolites of DHA fatty acids has been shown to ameliorate choroidal neovascularization in vivo (Yanai et al. 2014). While this suggests that sEH inhibition using small molecules would suppress CNV lesion vascular volume, it has not been previously investigated. Therefore, I assessed the effect of t-AUCB on CNV vascular volume using the L-CNV mouse model. Mice were intravitreally injected with different doses of t-AUCB immediately following laser application. Interestingly, t-AUCB dose-dependently suppressed CNV lesion vascular volume compared to vehicle (Figure 30). The effects at 10 and 1 µM final concentration of t-AUCB in the eye were comparable to 5 ng anti-VEGF164 control. Together these data suggest an important role of sEH in CNV formation.
Figure 30. sEH inhibitor t-AUCB dose-dependently suppressed CNV lesion vascular volume in the L-CNv model. A. Representative images from confocal microscopy of agglutinin stained CNV lesions, scale bars = 50 µm. D. Dose-dependent inhibition of the volume of CNV lesions by t-AUCB compared to vehicle control. Mean ± SEM, n=12 eyes/treatment. One-way ANOVA, Tukey’s post hoc tests, *P<0.05, **P<0.01, ***P<0.001.

C.6. SH-11037 Inhibits sEH Activity In Vivo in The L-CNv Model

After establishing SH-11037 binding and in vitro inhibition of sEH activity, followed by demonstrating the significance of sEH inhibition in the suppression of CNV lesion progression, it was crucial to assess whether SH-11037 suppresses L-CNv lesion vascular volume through the inhibition of sEH in vivo. Therefore, I
analyzed the lipid profiles of the retina/choroid layers from mice, 3 days after CNV induction and intravitreal injections of 10 µM SH-11037 or t-AUCB. DHA epoxy and dihydroxy metabolites’ levels were evaluated to investigate sEH activity in vivo. Interestingly, 19,20 EDP and its dihydroxy metabolite, 19,20-dihydroxydocosapentaenoic acid (DHDP) appeared to be the most affected DHA metabolites by sEH inhibition (Figure 31A). To get a clear understanding about sEH activity, the ratio of 19,20 EDP/DHDP was calculated (Figure 31B). Significantly suppressed ratio of 19,20 EDP/DHDP after laser induction compared to the no laser control strongly indicated the upregulation of sEH levels in the L-CNV model as observed with immunofluorescence (Figure 29E). Moreover, 19,20 EDP/DHDP ratio significantly increased after SH-11037 treatment compared to the vehicle treated controls indicating sEH inhibition in vivo.

Figure 31. Laser-induced upregulated sEH activity is significantly inhibited by SH-11037. A. Lipid profile of retina/choroid for DHA-related metabolites from L-CNV or control mice treated with vehicle, 10 µM t-AUCB, or 10 µM SH-11037. EDP:
epoxydocosapentaenoic acids, DHDP: dihydroxydocosapentaenoic acids. B. The ratio of 19,20 EDP/DHDP between different treatment conditions and no laser control mice indicates increased sEH levels/activity following laser induction compared to no laser control, **P<0.01, and a significant sEH inhibition by SH-11037, *P<0.05 vs. vehicle. Mean ± SEM, n=5 mice/treatment. One-way ANOVA, Dunnett’s post hoc test.

D. Discussion

Understanding the molecular mechanisms involved in abnormal neovascularization is crucial for the development of novel therapies for the treatment of ocular angiogenic diseases such as wet AMD. Currently, the gold standard, FDA approved treatments for wet AMD focus on inhibiting the VEGF signaling pathway, using biologics. Despite the success of these medications, resistant and refractory patient populations necessitate the discovery of new therapeutic targets (Falavarjani and Nguyen 2013, Shima et al. 2008, Lux et al. 2007). Consequently, broadening the drug pipeline with novel therapeutic agents that target different, VEGF-independent mechanisms of action is critical (Smith and Kaiser 2014). Therefore, I used a forward chemical genetics approach using the antiangiogenic compound, SH-11037, to identify its binding partners and potentially unravel new drug targets for angiogenesis.

In a subpopulation of human patients, anti-VEGF therapies can cause adverse effects in multiple organs including the eye. Some of these
complications, such as retinal detachment and loss of neural retinal cells, are related to the doses of anti-VEGF treatment given to the patient (Simo and Hernandez 2008); thus, lowering anti-VEGF doses while maintaining the therapeutic efficacy would be beneficial (Falavarjani and Nguyen 2013). Aflibercept, a fusion protein that consists of VEGF receptor-binding sequences fused to a segment of a human antibody backbone, is used in the clinic for the treatment of wet AMD (Stewart 2012a). Interestingly, SH-11037 produced combined effects with different concentrations of aflibercept in inhibiting the proliferation of HRECs, which were more pronounced than each treatment alone. Moreover, our in vivo data in the L-CNV model indicate that SH-11037 not only demonstrated a strong therapeutic potential for the amelioration of CNV as a single treatment, but also combined with the standard anti-VEGF164 antibody. Intriguingly, combining two individually inactive doses of SH-11037 and anti-VEGF164 produced a significant inhibition of L-CNV lesions that was comparable to the fully active dose of either treatment alone, without interfering with retinal function. SH-11037 and anti-VEGF combinations tested in vitro and in vivo appeared synergistic according to excess over HSA and Bliss additivity, two established methods of assessing synergy (Borisy et al. 2003), suggesting a VEGF-independent mode of action of SH-11037.

In order to further confirm the VEGF-independent mechanism of action of SH-11037 and determine its protein target, I used a series of biochemical in vitro and in vivo experiments, and determined sEH as a protein target for SH-11037,
mediating its antiangiogenic activity in vivo. I performed a photoaffinity based chromatographic technique using porcine brain as a rich source of proteins and identified sEH as a protein-binding partner of SH-11037. The limited expression level of sEH in retinal endothelial cells has been previously reported (Hu et al. 2014), which might suggest a different antiangiogenic target of SH-11037 in vitro. Like other natural products, SH-11037 likely exhibits polypharmacology. However, the substantial upregulation of sEH in L-CNV retinal sections along with SH-11037-mediated suppression of CNV vascular volume and sEH inhibition as demonstrated by lipid profiling strongly indicate that SH-11037 exerts its antiangiogenic activity in vivo at least partially through inhibition of sEH activity. Interestingly, although t-AUCB, a specific sEH inhibitor demonstrated a higher potency than SH-11037 in inhibiting sEH enzymatic activity in vitro, their effective doses in vivo in the L-CNV mouse model were comparable, and lipid profiling indicated a higher sEH inhibition by SH-11037 than t-AUCB in the eye.

The effect of ω-3 polyunsaturated fatty acids (PUFA) levels on choroidal neovascularization has been previously reported in vivo in the L-CNV model (Yanai et al. 2014) and in wet AMD patients (Merle et al. 2014). Interestingly, in the retina of sEH knockout mice, pronounced changes in the levels of 19,20-DHDP, but not the epoxy metabolites of DHA, compared to wild-type control mice were observed (Hu et al. 2014). Moreover, 19,20-DHDP was able to rescue the suppressed retinal angiogenesis phenotype observed with these mice (Hu et al. 2014), and intraperitoneal injections of 19,20-EDP suppressed CNV lesion
volume in the L-CNV mouse model (Yanai et al. 2014). These results are consistent with my findings.

While the explanation for the absence of changes in different EDPs after sEH inhibition or knockout is not clear, it is possible that the DHA epoxy 19,20-EDP and diol 19,20-DHDP metabolites and the activity of sEH are the major factors underlying the observed changes in retinal and choroidal angiogenesis rather than DHA levels on their own. Despite the role of DHA in the eye, the variability in the circulating levels of DHA among individuals suggests that dietary intake of ω-3 PUFA would not be sufficient alone for the prevention or treatment of wet AMD (Souied et al. 2013). Therefore, small molecule inhibition of sEH is an alternative therapeutic approach of significant interest for wet AMD patients to augment DHA epoxy metabolites levels with or without dietary supplementation.

In conclusion, these findings reveal a central role of sEH inhibition on the attenuation of CNV lesions in the L-CNV mouse model (Figure 32). Developing novel, ocular specific sEH-targeted therapies is an appealing approach to complement or combine with the existing anti-VEGF medications to overcome their limitations and tackle multiple angiogenesis signaling pathways for improved treatment of wet AMD. Additionally, the implication of sEH and its inhibition using small molecules such as SH-11037 could be further investigated in other ocular angiogenesis diseases including PDR and ROP.
Figure 32. Inhibition of sEH by small molecules such as SH-11037 attenuates CNV by enhancing the antiangiogenic and anti-inflammatory activities of EDP.
CHAPTER VI. DISCUSSION
A. Summary and Discussion

A.1. Ocular Angiogenesis: Current Therapeutic Approaches and Their Limitations

Neovascular eye diseases are a major cause of blindness throughout life. Between 6% and 18% of childhood blindness is attributable to retinopathy of prematurity (ROP) (Gilbert et al. 1997). The disease is estimated to cause vision loss in 1300 children a year in the USA, and severe visual impairment in a further 500 (Faia and Trese 2011). Meanwhile, proliferative diabetic retinopathy (PDR) affects an estimated 17 million adults of working age worldwide, or 7% of the growing diabetic population (Yau et al. 2012). Finally, almost 2 million elderly Americans are affected by “wet” age-related macular degeneration (AMD) (Fine et al. 2000a). Wet AMD has an estimated loss of productivity burden of $5.4 billion annually in the United States (Brown et al. 2005). These diseases are characterized by the development of new blood vessels in the eye: either retinal (in ROP and PDR) or choroidal neovascularization (CNV), where blood vessels grow into the sensory retina, causing hemorrhage and severe vision impairment (de Jong 2006).

The current FDA approved treatments for wet AMD aim at targeting the most dominant angiogenic mediator, the vascular endothelial growth factor (VEGF). The advent of intravitreal injections of anti-VEGF biologics such as ranibizumab, bevacizumab, and aflibercept revolutionized the management of wet AMD (Folk and Stone 2010, Prasad et al. 2010, Hanout et al. 2013).
However, the substantial fraction of non-responsive or refractory patients to anti-VEGF drugs has limited treatment options. Additionally, the association of anti-VEGF medications with ocular and systemic side effects caused by inhibiting such a major signaling pathway makes the development of alternative, VEGF-independent therapy an unmet necessity. Moreover, the elaboration of antiangiogenic small molecules would have the advantage over biologics of possible optimization for topical and oral routes of administration. This will overcome some of the severe but rare complications of the invasive intravitreal injections that are currently used, such as endophthalmitis and intraocular pressure elevation (Falavarjani and Nguyen 2013). Towards this end, I elucidated and characterized the toxicity, therapeutic potential and the mechanism of action of a novel antiangiogenic small molecule SH-11037, a synthetic derivative of the natural homoisoflavonoid cremastranone, as a potential therapy for wet AMD.

A.2. SH-11037 is A Potent Antiangiogenic Compound In Vitro and Ex Vivo in Disease-Relevant Systems

In our initial work, SH-11037 demonstrated a more potent antiproliferative activity compared to its parent compound, cremastranone, in inhibiting the proliferation of HRECs (a disease-relevant cell type) with a GI\textsubscript{50} of 55 nM. Additionally, SH-11037 showed about 10-fold more selectivity towards inhibiting the proliferation of HRECs over macrovascular endothelial cells, with negligible effects on other ocular cell lines, suggesting the absence of ocular off-target
effects (Chapter IV). After such an exciting observation, I sought to fully evaluate the antiangiogenic potential of SH-11037 in vitro, ex vivo and in vivo in CNV-relevant model systems. I tested the antiangiogenic activities of SH-11037 on HRECs as a type of ocular microvascular endothelial cells that represent those small vessels that are mostly affected by pathological neovascularization. Interestingly, SH-11037 significantly suppressed HREC proliferation, migration, and tube-formation capabilities in a concentration-dependent manner. This effect was not due to the induction of cell death by apoptosis as demonstrated by the absence of a significant number of apoptotic cells by immunofluorescence staining for cleaved caspase-3 and TUNEL, suggesting that SH-11037 might have a cytostatic rather than a cytotoxic effect on endothelial cells. Additionally, SH-11037 significantly blocked the formation of choroidal sprouts ex vivo. This assay provides a model for choroidal microvascular endothelial cells in the presence of their supporting microenvironment of macrophages and pericytes (Shao et al. 2013).

A.3. Development of A Novel Ellipsoid Quantification Method for The In Vivo Evaluation of CNV Lesions

The laser-induced choroidal neovascularization (L-CNV) mouse model is the most commonly used model in academia and industry to evaluate the pharmacological effects of new therapies for wet AMD (Lambert et al. 2013). In this model, a laser beam is focused on the back of the eye and burns are applied to induce focal CNV. The mice are then sacrificed 14 days post-laser, when
choroidal layers are isolated and stained with an endothelial cell marker to allow the three dimensional (3D) quantification of L-CNV lesion volume ex vivo.

While ex vivo lesion quantifications of L-CNV are robust and powerful and therefore considered the gold standard in the evaluation of L-CNV lesions, in vivo imaging analysis of lesions would allow longitudinal studies. Optical coherence tomography (OCT) is a non-invasive, in vivo imaging technique that is widely used for the clinical evaluation of different ocular pathologies including wet AMD (Jia et al. 2014). This technique generates high resolution, cross sectional images of the retina that allow in vivo monitoring of disease progression (Huang et al. 1991). While OCT has been extensively used in preclinical research in different animal models including L-CNV, 3D quantifications of lesions vascular volume have been solely dependent on the utilization of specialized software that is not necessarily available and compatible with different imaging systems (Giani et al. 2011, Berger et al. 2014, Hoerster et al. 2012). Therefore, I developed and characterized a novel, simple quantification method for 3D measurement of L-CNV lesions vascular volume to use for the in vivo evaluation of SH-11037 on CNV lesion progression.

I showed that a simple ellipsoid volume quantification allowed the in vivo monitoring and 3D evaluation of L-CNV lesion volume using the Micron III and InVivoVue, two of the most commonly used preclinical OCT imaging systems.
Moreover, an ellipsoid volume calculation provides rapid yet reproducible and comparable results to the standard ex vivo quantification method using the L-CNV model, with the sensitivity to detect different response of drug therapies in a platform-independent fashion (Chapter III). Therefore, OCT quantification using ellipsoid volume measurement was further used for the evaluation of SH-11037’s therapeutic potential in the L-CNV mouse model in vivo, together with the standard Z-stack confocal quantification ex vivo.

A.4. SH-11037 Demonstrates a Strong Antiangiogenic Potential In Vivo in The L-CNV Model Without Signs of Ocular Toxicity

Before evaluating the therapeutic potential of SH-11037 in the L-CNV model, I sought to elucidate signs of ocular toxicity that might be associated with intravitreal administration of SH-11037. Interestingly, a supratherapeutic dose of 100 µM final concentration of SH-11037 in the eye, which is about 10 fold higher than the doses used for in vivo efficacy assessment, was not associated with any short or long-term signs of retinal injury, apoptosis, or inflammation. Moreover, SH-11037 did not interfere with pre-existing retinal vasculature or with retinal function, suggesting that SH-11037 specifically targets actively proliferating endothelial cells as previously observed; this compound had little effect on the proliferation of other ocular cell types in vitro (Chapter IV).
The antiangiogenic activity of different compounds in vitro does not necessarily recapitulate their behavior in vivo in disease-relevant animal models (Chapter I). Currently, there are no FDA approved small molecules for the treatment of ocular angiogenic diseases. However, several small molecules with a promising potential for the treatment of wet AMD are currently in clinical trials such as OC-10X, vatalanib and squalamine (www.clinicaltrials.gov) (Pecen and Kaiser 2015). The natural aminosterol squalamine underwent several rigorous in vitro (Sills et al. 1998), ex vivo (Sills et al. 1998), and in vivo (Higgins et al. 2000, Ciulla et al. 2003) evaluation steps before proceeding to clinical trials. Similarly, extensive evaluation of the antiangiogenic potential of SH-11037 in CNV-relevant systems is crucial for it to proceed to clinical evaluation.

With the promising antiangiogenic potential of SH-11037 in vitro and ex vivo with the absence of signs of ocular toxicity in vivo, I evaluated the efficacy of intravitreal injections of SH-11037 in the L-CNV mouse model. SH-11037 dose-dependently suppressed L-CNV vascular volume when given as a single injection immediately after laser compared to vehicle treated mice, with the 10 and 1 µM effects comparable to 5 ng mouse anti-VEGF164 antibody. These effects were prominent 7 days post-laser by ellipsoid quantification of OCT images in vivo as well as 14 days post-laser from the agglutinin-stained choroidal flatmounts ex vivo. Moreover, in a more clinically relevant experimental design, 10 µM SH-11037 as well as 5 ng anti-VEGF164 significantly inhibited further lesion development when given as a single injection 5 days post-laser, a time
point where near-maximal CNV lesion sizes are observed. While this indicates that both SH-11037 and anti-VEGF are successful in blocking further growth of CNV lesions but not in resolving pre-existing ones, this might be consistent with the irreversible damage caused by CNV progression in patients after which they are unresponsive to the standard-of-care anti-VEGF medications (Lux et al. 2007).

A.5. SH-11037 Synergizes with Anti-VEGF Therapies In Vitro and In Vivo

In some wet AMD patients, complications of anti-VEGF therapeutic approaches such as retinal detachment have been related to the doses of anti-VEGF treatment given to the patient (Simo and Hernandez 2008). Therefore, reducing the doses of anti-VEGF medications while maintaining the therapeutic efficacy would be beneficial (Falavarjani and Nguyen 2013). I tested combinations of SH-11037 and anti-VEGF in vitro and in vivo. Aflibercept, a fusion protein of VEGF receptor-binding sequences fused to a segment of a human antibody backbone, that is widely used in the clinic for the treatment of wet AMD (Stewart 2012a) was used in vitro on HRECs, and the mouse anti-VEGF164 antibody was used in vivo in the L-CNV model. The mouse anti-VEGF164 antibody is a murine-optimized equivalent of bevacizumab, the standard of care in humans.
Interestingly, SH-11037 showed pronounced combined effects with different aflibercept concentrations in inhibiting the proliferation of HRECs. Moreover, combining two individually inactive doses of SH-11037 and anti-VEGF164 significantly suppressed lesion vascular volume in the L-CNV model, which was comparable to the fully active dose of either treatment alone, without interfering with retinal function. Intriguingly, the synergistic SH-11037/anti-VEGF combinations in vitro and in vivo do not only indicate the possibility of minimizing anti-VEGF limitation without affecting the therapeutic efficacy, but also indicates a VEGF-independent mode of action of SH-11037.

Combination therapies are being intensely investigated in multifactorial diseases such as wet AMD to tackle different disease components, minimize dosing frequency, and to improve the response of patients who do not respond to monotherapy. Photodynamic therapy (PDT) is a method in which intravenous administration of a photosensitive dye, verteporfin, which preferentially concentrates in newly formed blood vessels such as those in CNV, will seal off the leaky vasculature after activation by non-thermal laser (Arnold and Heriot 2007). PDT is no longer used as monotherapy since it is less efficacious than anti-VEGF treatments and does not improve vision (Jager et al. 2008). Interestingly, using PDT in combination with anti-VEGF and steroids has been extensively tested in clinical trials and shown to result in enhanced efficacy in improving vision. Moreover, not only dual PDT/anti-VEGF or PDT/steroids are being evaluated, but also triple therapy of PDT to eradicate existing CNV, anti-
VEGF to inhibit angiogenesis and steroids to suppress inflammation are currently under investigation for their superior potential for future use in the clinic for wet AMD patients (Augustin et al. 2007, Englander and Kaiser 2013). This currently increasing interest in combination therapies greatly supports the potential of small molecules that target VEGF-independent mechanisms such as SH-11037 to combine with anti-VEGF biologics for enhancing the efficacy and improving vision in wet AMD patients.

A.6. SH-11037 Inhibits sEH In Vitro and In Vivo Mediating a VEGF-Independent Antiangiogenic Effect

With such a strong antiangiogenic potential of SH-11037 in various CNV-relevant systems in vitro and in vivo, elucidation of the mechanism of action of SH-11037 is critical. Using a series of biochemical approaches in vitro and in vivo, I characterized soluble epoxide hydrolase (sEH) as a protein target that is potentially mediating the antiangiogenic mechanisms of SH-11037 in vivo. sEH is a 62 kDa bifunctional enzyme with an N-terminal lipid phosphatase activity, whose function is not fully understood, and a C-terminal epoxide hydrolase activity, which is involved in the metabolism of ω-3 and ω-6 polyunsaturated fatty acids (PUFA) (Harris and Hammock 2013). It is encoded by the EPHX2 gene with 555 amino acid residues in humans and 554 residues in mice, and exists as homodimer in the cytosol (Nelson et al. 2013). It is expressed in multiple human tissues, with the highest expression seen in the liver. The epoxide hydrolase activity of sEH has been extensively studied for its role in the metabolism of the
ω-6 arachidonic acids’ epoxide intermediates, epoxyeicosatrienoic acids (EETs) (Figure 33) (Panigrahy et al. 2011, Zhang et al. 2014). Due to the role of EETs in ameliorating hypertension, pain and inflammation, sEH inhibitors are being evaluated in clinical trials for their therapeutic potential in cardiovascular protection (Morisseau and Hammock 2013). Meanwhile, sEH is also involved in the metabolism of ω-3 fatty acids, docosahexaenoic acids (DHA) and eicosapentaenoic acids (EPA) epoxides (Harris and Hammock 2013).

Figure 33. The role of soluble epoxide hydrolase in the metabolism of ω-3 and ω-6 fatty acids.

While the role of DHA and EPA epoxy metabolites’ levels on choroidal neovascularization has been previously reported in vivo in the L-CNV model
(Yanai et al. 2014) and in wet AMD patients (Merle et al. 2014), the direct evaluation of sEH levels and inhibition using small molecules in the L-CNV model have not been previously tested. A potent and specific sEH inhibitor, t-AUCB demonstrated a dose-dependent inhibition of CNV lesion volumes when delivered intravitreally. Moreover, sEH levels were significantly upregulated in the L-CNV treated mice compared to untreated controls, indicating an important possibility of sEH inhibition in ameliorating CNV in vivo.

Lipid profiling of retina/choroid layers demonstrated that SH-11037 inhibited sEH in vivo in the L-CNV model, as indicated by an increased ratio of epoxy/diol metabolites of DHA, particularly 19,20-EDP/DHDP. Similar effects were observed with t-AUCB. Interestingly, 19,20-EDP has been previously shown to ameliorate retinal and choroidal neovascularization in vivo (Hu et al. 2014, Yanai et al. 2014). These data indicate that SH-11037-mediated suppression of CNV vascular volume was likely due to sEH inhibition. Interestingly, despite the higher potency of t-AUCB compared to SH-11037 in inhibiting sEH enzymatic activity in vitro, their effective doses in vivo in the L-CNV mouse model were comparable. Additionally, lipid profiling surprisingly indicated higher sEH inhibition by SH-11037 than t-AUCB tested at the same dose of 10 µM.
A.7. Inhibition of sEH Using Small Molecules Such as SH-11037 has Potential for The Treatment of Wet AMD

Wet AMD is a multifactorial disease that involves genetics and environmental factors including smoking, obesity and nutrition (Lim et al. 2012). Long chain PUFA, such as EPA and DHA, have vital roles in the structure and function of various organs including the eye. PUFA are an important source of precursors of signaling molecules that regulate various cellular functions such as inflammation and angiogenesis. Epoxy fatty acids have been shown to attenuate inflammation via the activation of peroxisome proliferator-activated receptor-γ (PPARγ), nuclear receptors that regulate fatty acid storage and metabolism, with the consequent inhibition of NF-κB activity and expression of downstream genes (Stahl et al. 2010). Interestingly, the ω-3 fatty acid DHA has been shown to be the major PUFA in the sensory and vascular retina with the highest body concentrations of DHA per unit weight found in phospholipids of retinal photoreceptor outer segments (SanGiovanni and Chew 2005). Although the high unsaturation of PUFAs of membrane phospholipids predisposes them for lipid peroxidation with the production of cytotoxic compounds, the dietary insufficiency of these fatty acids is associated with pathological alteration of visual system structure and function (SanGiovanni and Chew 2005).

Since inflammation and angiogenesis play a pivotal role in wet AMD, the antiinflammatory and antiangiogenic properties of DHA and EPA made them of particular interest. Indeed, studies have shown that dietary intake of ω-3 fatty
acids are associated with a significantly reduced risk of AMD progression (Robman et al. 2007, SanGiovanni et al. 2008, Tan et al. 2009, SanGiovanni et al. 2009). However, the variability in the circulating levels of DHA and EPA among individuals and the variability in dietary assessment suggest that dietary intake of ω-3 PUFA would not be sufficient alone for the prevention or treatment of wet AMD (Souied et al. 2013). Therefore, given the key role of sEH in the metabolism of DHA, inhibiting sEH using small molecules such as SH-11037 would greatly enhance DHA levels in the eye with possible beneficial antiangiogenic and anti-inflammatory effects.

Overall, this study demonstrates the safety and therapeutic potential of SH-11037 as a novel sEH inhibitor with potential for the treatment of wet AMD (Figure 34). Additionally, it will be valuable to develop novel SH-11037-based inhibitors of sEH for treating the debilitating ocular diseases caused by neovascularization such as wet AMD.
B. Limitations of The Study

While no major difficulties were encountered in this study, I would like to consider a few potential limitations. Given the commercial unavailability and limitations in obtaining human choroidal endothelial cells, a CNV-relevant cell type, I used HRECs in the in vitro evaluation of SH-11037. The choice of HRECs was based on the several similarities between choroidal and retinal endothelial cells: they are both ocular and microvascular in nature. However, there are also some distinct differences that need to be taken into consideration, such as the fenestration of choroidal endothelial cells and the physiological differences between retinal and choroidal capillary beds and their vascular supply (Stewart et
Retinal circulation is characterized by high oxygen extraction and low blood flow, while the choroidal circulation exhibits a high blood flow and low oxygen extraction capability (Campochiaro 2000). The retinal circulation is mainly controlled by autoregulation under the influence of local factors; meanwhile, the choroidal circulation is controlled by sympathetic innervation (Delaey and Van De Voorde 2000). The different properties of choroidal and retinal endothelial cells are also indicated by their preferential susceptibility to certain diseases such as diabetic retinopathy versus wet AMD, respectively, due to their different characteristics as well as varying needs of the tissues in which they exist (Stewart et al. 2011). While using commercially available bovine choroidal endothelial cells might be an alternative, it is widely accepted that endothelial cells from different species possess different morphological, biochemical and phenotypic heterogeneity (Aird 2003, Fajardo 1989). This suggests that the use of bovine endothelial cells may not be truly representative of the pathological mechanism of wet AMD. Therefore, I opted to use HRECs, being a closer representative of human ocular microvascular endothelial cells.

In this study, I used the L-CNV model for the in vivo evaluation of SH-11037 because it is the most commonly used and widely accepted animal model in academia and industry for the study of wet AMD and evaluation of potential therapies. However, it is important to consider the shortcomings of this model given the absence of a macula in mice and the artificial nature of this model (Lambert et al. 2013). While the L-CNV model does not mimic the complexity of
wet AMD pathology, it recapitulates the CNV characteristics of wet AMD with its inflammation and angiogenesis features. Other, genetically engineered animal models are available for the study of different aspects of wet AMD pathogenesis such as the complement factor H knockout model. However, these models are mainly used for elucidating the role of various mediators in the development and progression of AMD, and they do not involve CNV formation (Pennesi et al. 2012). Other models that spontaneously develop CNV such as transgenic mice overexpressing VEGF164 in the RPE have the disadvantage of the length of time required for the development of CNV. Despite the limitations of the L-CNV model, it has been used for preclinical evaluation of the therapeutic effects of drugs in patients with wet AMD, such as with the “VEGF-trap”, aflibercept (Saishin et al. 2003, Heier et al. 2012). Therefore I used the L-CNV mouse model for the evaluation of SH-11037’s antiangiogenic potential in vivo.

As a positive control for the L-CNV mouse model in vivo I used mouse anti-VEGF164 neutralizing antibody (R&D Systems). Intravitreal injections of the mouse anti-VEGF164 antibody have been used previously in this model in some studies (Liu, Qi, et al. 2013), while others used bevacizumab (Avastin), a human VEGF antibody that is commonly used in the clinic for wet AMD treatment (Davis et al. 2012). It has been previously shown that bevacizumab is optimized for targeting human VEGF signaling. It is not as efficient in blocking murine VEGF signaling (Ferrara and Adamis 2016). Additionally, a recent study (Irani et al. 2016) showed that bevacizumab bound strongly to human VEGF-A, but showed
5-log weaker binding to both mouse and rat VEGF-A, due to a single amino acid substitution in the bevacizumab binding site. Therefore, in order to be able to use minimal doses needed to ensure specificity, while maintaining the anti-angiogenic effect of anti-VEGF, I used the mouse anti-VEGF164 neutralizing antibody for the L-CNV mouse model.

Another limitation of this study is the use of porcine brain in the photoaffinity pulldown approach to reveal the protein targets of SH-11037. While one alternative was to use HRECs, the amount of protein obtainable from HREC cell lysates was not enough for pulldown identification in preliminary experiments. Another option was to use bovine eyes, since they are more representative of the variety of proteins present in human eyes. Unfortunately, the high levels of connective tissue and the presence of thick sclera made the retina/choroid/sclera homogenate diluted with non-relevant material that resulted in inconsistent observations. Therefore, I performed the pulldown assays using porcine brain, which is a rich source of protein to ensure that I would initially have enough protein for detecting low-abundance targets. The identified protein targets required further validation for their relevance in ocular angiogenesis by using HRECs. Interestingly, the identified sEH enzyme has low expression levels in HRECs, as previously noted (Hu et al. 2014), and as demonstrated by immunofluorescence, immunoblot, and mRNA quantification. While this observation limited the possibility of the target relevance to SH-11037-mediated antiangiogenic activity in vitro, sEH relevance and validation to CNV in vivo was
possible using the L-CNV mouse model. Overall, this might indicate polypharmacology of SH-11037 with different in vitro and in vivo targets, but it also indicates an sEH-mediated antiangiogenic mode of action in vivo, which is more relevant as a preclinical model evaluation. Further optimization of SH-11037 would be necessary to increase the specificity to sEH inhibition.

C. Future Directions

The outcomes of this study are an understanding of the mechanism of action of the novel antiangiogenic compound SH-11037 (Chapter V), together with evaluation of its efficacy and toxicity in a preclinical model of CNV (Chapter IV), and development of a novel analytical method for this model (Chapter III). Since a binding partner of SH-11037 is sEH, SH-11037 could be used as a lead for the development of antiangiogenic drugs to prevent blindness associated with wet AMD. The completion of this project opens up a number of proposed future studies.

Given the in vitro and in vivo inhibition of sEH by SH-11037, a full understanding of the mode of inhibition and determination of SH-11037 inhibitory potency compared to the specific sEH inhibitor t-AUCB is important to investigate. Dimerization of sEH has been shown to be required for its hydrolase activity (Nelson et al. 2013). Whether SH-11037 interferes with sEH dimerization, or binds to the catalytic site of sEH similar to t-AUCB, has not been yet explored. Molecular docking of SH-11037 and sEH would not only be beneficial to predict
the binding mode of SH-11037 and compare it to t-AUCB binding, but also to further optimize SH-11037’s structure to enhance its binding and inhibition of sEH.

The development of structurally modified analogs of SH-11037 with higher potency and better solubility while still inhibiting sEH is a critical next step in the process of introducing SH-11037 for clinical studies. Given the limited water solubility of SH-11037, an oral route of administration might not be suitable. However, as a small molecule, SH-11037 could potentially be optimized for topical applications, which would be appealing as a non-invasive local delivery route, while still minimizing the side effects of systemic administration. Topical delivery could also enable frequent dosing if necessary. Alternatively, delayed-release formulations of this small molecule therapy for intravitreal injection might be possible. Determination of the pharmacokinetics of SH-11037 is therefore an essential step to further improve SH-11037 efficacy and route of administration.

In a previous study, SH-11037 demonstrated a significant inhibition of retinal neovascularization in the oxygen-induced retinopathy (OIR) mouse model of retinopathy of prematurity (ROP) (Basavarajappa et al. 2015). However, the exact mechanism by which SH-11037 produced such an antiangiogenic effect has not been yet tested. Therefore, lipid profiling and evaluation of potential sEH inhibition in the OIR model together with examining the potential of SH-11037 and anti-VEGF combinations in the amelioration of retinal angiogenesis would be
crucial. Additionally, further studies of the in vivo effects and mechanism of SH-11037 could be done with animal models of other neovascular eye diseases such as proliferative diabetic retinopathy (PDR).

Neovascularization is not only implicated in ocular angiogenic eye diseases but also in various diseases including cancer. Retinoblastoma is a cancer of the developing retina that is responsible for 1% of childhood cancer deaths and 5% of childhood blindness worldwide (Dimaras et al. 2012, Rodriguez-Galindo et al. 2015). Treatment approaches for retinoblastoma range from laser ablation focal therapy, to systemic chemotherapy to enucleation in the case of large tumors to salvage life. There is thus a strong need to develop novel pharmacotherapies that could be delivered locally to specifically control the progression of retinoblastoma to save vision, eyes, and lives. Retinoblastoma tumors produce pro-angiogenic factors and it has been shown that retinoblastoma angiogenesis is associated with a high risk of metastasis (Albert et al. 1984, Tapper et al. 1979, Marback et al. 2003). Therefore, inhibiting angiogenesis might be an appealing target for the treatment of retinoblastoma. Given the demonstrated strong antiangiogenic potential of SH-11037 presented in this study, testing the therapeutic potential of SH-11037 in controlling tumor progression in a preclinical mouse retinoblastoma model as well as models of other neoplastic diseases is worthy of investigation.
The completion of this study characterized SH-11037 as a potent antiangiogenic small molecule with a therapeutic potential for the in vivo amelioration of CNV without causing ocular toxicity. The exciting advantage of the VEGF-independent, sEH-mediated mechanism of SH-11037 opens up various opportunities for further investigation of the role of sEH in ocular disease pathology, as well as the therapeutic potential of SH-11037 in other neovascular diseases of the eye and other tissues including cancer.
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- Biochemistry diploma
PUBLICATIONS

• Sulaiman RS, Merrigan S., Quigley J, Qi X, Lee B, Boulton ME, Kennedy B, Seo SY, Corson TW. A novel small molecule ameliorates ocular neovascularisation and synergises with anti-VEGF therapy. Scientific Reports. 2016, 6, 25509

• O’Hare M, Shadmand M, Sulaiman RS, Sishtla K., Sakisaka T, Corson TW. Kif14 overexpression accelerates murine retinoblastoma development. International Journal of Cancer, DOI: 10.1002/ijc.30221

• Sulaiman RS, Quigley J, Qi X, O’Hare MN, Grant MB, Boulton ME, Corson TW. A simple optical coherence tomography quantification method for choroidal neovascularization. Journal of Ocular Pharmacology and Therapeutics, 2015, 31 (8), 447-454


• Sulaiman RS, Basavarajappa HD, Corson TW. Natural product inhibitors of ocular angiogenesis. Experimental Eye Research, 2014, 129, 161-171

• Lee B, Basavarajappa, HD, Sulaiman RS, Fei X, Seo SY, Corson TW. The first synthesis of the antiangiogenic homoisoflavanone, cremastranone. Organic and Biomolecular Chemistry. 2014, 12 (39), 7673 - 7677
PUBLISHED ABSTRACTS


• Sulaiman RS, Basavarajappa HD, Lee B, Fei X, Seo SY, Corson TW. A small molecule pharmacotherapy ameliorates laser-induced choroidal neovascularization. Investigative ophthalmology & visual science. 2015; 56: E-abstract 2470

• Basavarajappa HD, Qi X, Sulaiman R, Lee B, Quigley J, Sishtla K, Shadmand M, Boulton ME, Seo SY, Corson TW. Ferrochelatase is a novel mediator of ocular angiogenesis. Investigative ophthalmology & visual science. 2015; 56: E-abstract 5854

PRESENTATIONS


- **Sulaiman RS**, Basavarajappa HD, Lee B, Fei X, Seo SY, Corson TW. An anti-angiogenic small molecule therapy for choroidal neovascularization. The 12th congress on ocular pharmacology and therapeutics (AOPT). February 2015, Charleston, SC. *Oral presentation*


PATENTS

PROFESSIONAL MEMBERSHIPS

2015- Present  Member, The Association of Research in Vision and
Ophthalmology (ARVO)

2015- Present  Member, The Association of Ocular Pharmacology and
Therapeutics (AOPT)

2012-2013  Member, Society for Neuroscience (SfN)

2006-2011  Member, Egyptian Pharmacy Syndicate (EPS)

FELLOWSHIPS AND AWARDS

• **K.K. Chen Fellowship in Pharmacology and Toxicology.** Indiana
University School of Medicine – “In recognition of outstanding scholarship,
innovative research, and exemplary dedication to the spirit of scientific
investigation” – May 2016

• **IUSM Graduate Student Travel Award.** Indiana University School of
Medicine – April 2016

• **Knights Templar Travel Award.** Association of Research in Vision and
Ophthalmology (ARVO) – May 2015

• **Hot Topic Distinction Award.** Awarded to the top 3% of poster
presentations - Association of Research in Vision and Ophthalmology
(ARVO) – May 2015
• **Paradise Travel Award.** Department of Pharmacology and Toxicology, IUSM – *May 2015*

• **Best Young Investigator Abstract Award.** Association of Ocular Pharmacology and Therapeutics (AOPT) – *February 2015*

• **MaryAnn Liebert Travel Award.** Association of Ocular Pharmacology and Therapeutics (AOPT) – *February 2015*

• **Indiana University School of Medicine fellowship.** For PhD graduate students. *2011-2012*

• **Eva pharmaceuticals Award, Egypt.** For Top 20 Pharmacy graduates with honors – *April 2007*

• **Egyptian Pharmacy Syndicate Award, Egypt.** For Top 10 Pharmacy graduates – *October 2006*

**EXTRACURRICULAR ACTIVITIES**

**Lecturer for prospective PhD applicants**

*May 2013 - present*

• Mentoring prospective graduate students on PhD application process and volunteering personal statement and application material revision for PhD applicants – Number of applications revised: 20

• Organizing lectures on “How to apply for a PhD in the US” (in Arabic)

YouTube link: https://www.youtube.com/watch?v=5xBwwcodPog (4 parts)
Covering tips on writing a very competitive personal statement and getting strong recommendation letters

**Tutor - Biochemistry and molecular biology**

*April 2007 – January 2011*

Tutoring pharmacy school undergraduates on various biochemistry courses and laboratory techniques including:

- Biochemistry I, II, III (pharmaceutical sciences undergraduates)
- Biochemistry and Molecular Biology (undergraduate clinical pharmacy program)

Number of students tutored: 15