EVALUATION OF HIF INHIBITORS FOR THE TREATMENT OF OCULAR NEOVASCULAR DISEASES

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EVALUATION OF HIF INHIBITORS FOR THE TREATMENT OF OCULAR NEOVASCULAR DISEASES

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ABSTRACT

Hypoxia-inducible-factor (HIF)-mediated expression of pro-angiogenic genes under hypoxia is the fundamental cause of pathological neovascularization in ocular diseases and cancers. Thus, the inhibitors of the HIF pathway or key regulators such as histone demethylases (KDMs), that amplify HIF signaling, can have profound therapeutic value for these diseases.

This dissertation reports findings about phytochemical molecules - honokiol and berberine and shows them to be potent inhibitors of the HIF pathway as well as hypoxia-induced expression of KDMs in a number of cancer and retinal pigment epithelial cell lines. The results provide an evidence-based scientific explanation for therapeutic benefits observed with honokiol and berberine. On comparing the anti-HIF effect of both these compounds honokiol was found to possess a better efficacy that exhibited it's effect on the HIF pathway by inhibiting HIF binding to the HRE. Further in this study honokiol was compared with two other recently identified HIF inhibitors from natural sources - digoxin and doxorubicin. The results show that honokiol has a better safety to efficacy profile as a HIF inhibitor than digoxin and doxorubicin. Therefore honokiol was chosen as candidate molecule for further clinical evaluation for the treatment of pathological ocular neovascularization.
Honokiol inhibited hypoxic - mediated secretion of VEGF by retinal pigment epithelial cells. Hence honokiol’s anti-angiogenic activity was evaluated using various in vitro angiogenesis assays that mimic ocular neovascularization. A robust inhibition of human retinal micro vascular endothelial cell (hRMVEC) activation and proliferation was observed with honokiol treatment. Also, hRMVEC cells lost their ability to migrate and form well-networked tubes essential for pathological ocular neovascularization. Further, it is shown for the first time that daily intraperitoneally injection of honokiol starting at postnatal day (P) 12 in an oxygen-induced retinopathy (OIR) mouse model significantly reduced retinal neovascularization at P17. Administration of honokiol also prevents the oxygen-induced central retinal vaso-obliteration a characteristic feature of the OIR model. Honokiol also enhanced physiological revascularization of the retinal vascular plexuses. Since honokiol suppresses multiple pathways activated by HIF, in addition to the VEGF signaling, it may provide advantages over current treatments utilizing specific VEGF antagonists for ocular neovascular diseases and cancers.
APPROVAL PAGE

The undersigned, appointed by the Dean of the School of Graduate Studies have examined a dissertation titled “Evaluation Of HIF Inhibitors For The Treatment of Ocular Neovascular Diseases”, presented by Divya Teja Vavilala, candidate for the Doctor of Philosophy degree, and hereby certify that in their opinion it is worthy of acceptance.

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DEDICATED TO MY PARENTS AND MY HUSBAND
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CHAPTER I
INTRODUCTION AND LITERATURE REVIEW

Hypoxia

During the course of evolution, the paleoproterozoic era saw the generation of free oxygen. Over the period of time, levels of free oxygen and oxygen dissolved in the oceans started increasing in the atmosphere [32]. This change in the dioxygen (O₂) form of oxygen led to the evolution of aerobic species and thereby Homo sapiens. Oxygen in our body is employed as the terminal electron acceptor in oxidative phosphorylation. Oxidative Phosphorylation is a key process that is involved in generation of the major energy molecule of the cell- Adenosine Triphosphate (ATP). Apart from this, oxygen in our body also plays a role in commencement of apoptosis [33], membrane transport regulation [34], gene expression of oxygen dependent genes [35] and in various other processes. Having briefly highlighted the importance of oxygen in our body, it becomes clear that it is very essential to maintain a threshold of oxygen level. If the oxygen tension in our body drops below what is required, cells experience hypoxia. Hypoxia is seen both in pathological as well as physiological conditions. Physiological processes where hypoxia is experienced include wound healing, embryonic development, and higher altitudes. Pathological conditions are cancer, ischemia, ocular neovascularization, and anemia. [36-38].

Under hypoxia, cells undergo a diverse array of signal transduction pathways to maintain oxygen homeostasis and cellular metabolism. This adaptation is observed both at the cellular as well as the systemic level. ATP production shifts from oxidative phosphorylation to glycolysis, oxygen supply to the cells is increased, formation of new
blood vessels (*i.e.* angiogenesis) is observed etc. [39-41]. To achieve such an orchestrated adaptation to hypoxia at various levels many pathways like Hypoxia Inducible Factor (HIF), Nuclear Factor-κB (NF-κB), Mammalian Target of Rapamycin (mTOR), and Endoplasmic Reticulum Stress pathway act in a concerted fashion.

**Hypoxia Inducible Factor (HIF) Pathway**

Under hypoxia, hypoxia-inducible factor – (HIF) is the master regulator of transcription of more than 100 genes [42]. It is really important to understand different aspects of HIF *i.e.* its structure, regulation, due its role played in pathophysiological conditions such as cancer, ischemia, ocular neovascularization, and anemia.

HIF is a heterodimer that is comprised of HIF-α and HIF-β. HIF-β and HIFα are constitutively expressed but the stability of HIF-α in the cell is oxygen dependent [43]. HIFα has three isoforms (*i.e.* HIF-1α, HIF-2α and HIF-3α) [44] (Figure 1). HIF-1α shows pervasive expression in all cells while HIF-2α displays tissue-specific expression as in endothelial cells in the hippocampal region, glomerular endothelial cells in kidney and in hepatocytes. [45]. Though HIF-3α is also known to dimerize with HIF-β to activate transcription [46] it is unique from the other HIF-α isoforms as it is subjected to extensive splicing. HIF-3α has about 10 splice variants [47]. One of the HIF-3α splice variant is an inhibitory PAS (IPAS) domain protein that acts as a negative regulator of HIF-1α [48]. HIF-β expression is seen to be high in brain and in kidney. The role of HIF-β as a transcription factor is not only limited to hypoxia. It forms a heterodimer with HIF-β, which is a dioxin receptor and is involved in responses to dioxin.[49]

Common domains that are present in both HIF-α and HIF-β are the bHLH domain (basic helix loop helix) and PAS domain. The bHLH domain is involved in the DNA
binding. PAS domain is named based on its identification in PER, ARNT and SIM proteins and is involved in dimerization. Proteins that have the PAS domain fall under the PAS superfamily. These proteins are mostly found in prokaryotes and involved in responses to external stimuli [50].

Except for HIF-3α all the other HIF α and β subunits have a C-terminal transactivation domain (CTAD). The role of C-terminal transactivation domain is not yet clear for HIF-β subunits but in HIF-α, it plays a role in the recruitment of different transcription coactivators for the formation of a transcription initiation complex. It has been reported that HIF-1α splice variants that lack exon 11 and 12 or exon 14 have no or reduced transactivation activity [51]. The N-terminal transactivation domain is a common feature present in all the HIF-α subunits. This domain might either overlap or adjoin the oxygen dependent degradation domain (ODD). CTAD and ODD act as a harbor for posttranslational modifications of HIF-α. These modifications act as signals for the oxygen dependent HIF-1α stability and activity [43, 52-56].
Figure 1: Cartoon representation of HIF-α (HIF-1α, HIF-2α, HIF-3α, and IPAS) and HIF-β showing different protein domains. The length of each protein is represented on the side.
Under normoxia, the ODD of HIF-1α is hydroxylated by prolyl hydroxylases (PHD1, PHD2 and PHD3) [57]. Hydroxylation provides an arm for the p-VHL to bind to HIF-1α ODD [58]. This leads to recruitment of proteins that ubiquitinate HIF-1α followed by proteosomal degradation of HIF-1α[48]. Under hypoxia PHDs become impaired by lack of their co factors (*i.e.* oxygen and Fe$^{2+}$) and loose their capacity to hydroxylate HIF-1α. Hence HIF-1α escapes proteosomal degradation. An NLS sequence present in HIF-1α on the C-terminus, binds to the nuclear pore proteins and thereby translocates it into the nucleus [59].

Additional asparaginyl hydroxylation by Factor Inhibiting HIF (FIH), on the CTAD renders HIF transcriptionally inactive. This prevents HIF binding by its coactivators (*i.e.* p300 and CREB binding proteins (CBP)) [60-62]. *In vitro* studies show that PHD has a three times higher $K_m$ for oxygen when compared to FIH. Hence for HIF to be fully active a further drop in the oxygen concentration is required [63]. Once HIF heterodimer gets activated it binds to hypoxia- responsive elements (HREs) present in the promoter, enhancer and or in the intron regions of the target genes [64-66].

The meaning of minimal Hypoxia Response Elements (HRE) still remains to be defined. A simple model of HIF heterodimer binding to the HREs and causing transactivation of the genes responsible for the adaptive response to hypoxia does not yet exist. HREs are *cis*-regulatory elements, which are present in the enhancers or promotors of the target genes. It is comprised of a conserved A/GCGTG sequence, which acts as the HIF binding sequence (HBS). HBSs are oriented even in antiparallel fashion in a few functional HREs (e.g. in *transferrin* [67], murine *PGK-1* and *LDHA* genes [68]); and it forms the core of the HRE while the flanking sequence is variable. The core sequence of HBS is found even in a few non-functional HREs. A requirement for the presence of the core sequence of the HBS in
functional HREs is not yet clear. There might be plausible differences in the variable flanking sequences of functional and non-functional HREs [66]. In a few of the genes a HIF ancillary site was found that aids in the HIF binding to the HBS [69]. Mutation studies at three different sites in the murine LDHA promoter abrogated hypoxic induction of the gene. But when these sites were concatamerized it was not sufficient for the hypoxic gene induction [68]. This revealed a much more complex level of regulation. CpG island methylation state is also considered to play a selective role in the gene regulation. The core HBS that contains the CpG dinucleotide can be methylated by DNA methyltransferases. It is observed that HIF does not bind to the hemimethylated CpG, while methylated CpG of erythropoietin HBS plays a role in its gene regulation [70].

HBS-bound HIF also binds to adjacently placed transcription factors. These factors play a role in either tissue specific hypoxic response or they aid in the hypoxic response [66]. For example, HIF binds to AP-1 binding factors in the VEGF promoter [71], hepatic nuclear factor 4 in the EPO enhancer [72], ATF-1 and CREB-1 in the murine LDHA promoter [73].

To date nothing decisive has turned out from all the studies regarding the design of minimal HREs. However multimers of HREs of varying length from genes like VEGF and EPO have been used in the construction of hypoxia inducible vectors [74, 75]. HIF binding to HRE regulates transcription of hypoxia response genes involved various cellular processes that help in hypoxic adaptation. These are gene involved in angiogenesis, glucose metabolism, cell proliferation and survival etc (Figure 2).
## Hypoxia Inducible Factor (HIF) target genes

<table>
<thead>
<tr>
<th>Glucose metabolism</th>
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<tr>
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<tr>
<td>Hexokinase-1,2 [12, 17]</td>
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<td>Phosphofructokinase L [23]</td>
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<td>6-Phosphofructo-2-kinase/fructose-2,6-biphosphate-3 [31]</td>
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<td>Transforming growth factor-β3 [18]</td>
<td>IGF binding protein 1-3 [19]</td>
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<td>Angiopoietin 2 [21]</td>
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<td>Stromal derived growth factor 1 [24]</td>
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<td>JARID1B [7]</td>
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</table>

**Figure 2:** Classification of genes regulated by HIF
Regulation of HIF

Translational regulation of HIF-1α mRNA

Under normoxia HIF-1α mRNA undergoes cap dependent translation. HIF-1α mRNA is translated by the trigger of different cellular signals like cytokines, growth factors and oncoproteins [65]. These signals activate mitogen activated protein kinase (MAPK) and phosphatidylinositol 3-kinase (PtdIns3K-Akt) mTOR pathways [76, 77].

During hypoxia an inhibition of cap dependent mechanisms is observed; hence HIF-1α mRNA is translated by a cap independent mechanism. Shutdown of cap dependent translation stops the protein synthesis of other proteins as well. Two pathways facilitate this shutdown [78, 79]. Under acute hypoxia Unfolded Protein Response (UPR) gets activated in less than 2 hr. The downstream target pathway of UPR is PKR like ER Kinase (PERK). The PERK pathway phosphorylates eIF2a that prevents the formation of translation initiation complex [80]. The second pathway for translational shutdown is the mTOR regulated pathway which acts under longer exposure to hypoxia. Under hypoxia, mTOR is negatively regulated by – 1) tuberous sclerosis [TSC 1 and 2] and 2) regulated in development and DNA-damage responses (REDD1). This lead to hypophosphorylated substrates of mTOR – 1) S6K a ribosomal kinase and 2) eukaryotic initiation factors (eIF) 4E-binding proteins [81-83].

S6K phosphorylation is essential for phosphorylation of the downstream proteins like S6 (ribosomal protein), eIF4B, programmed cell death 4(PCDC4), eukaryotic elongation factor 2 kinase (eEF2K), cap-binding protein (CBP80), and S6K1 Aly/REF-like target (SKAR) [84]. mTOR’s second substrate, (eIF) 4E binding proteins (eIF4E-BP1), upon phosphorylation does not form a complex with eIF4E. Hence eIF4E is free to bind to eIF4F
subsequent translational initiation. Hypophosphorylated eIF4E-BP1 has higher binding affinity for eIF4E thereby preventing eIF4E complex formation and cap dependent translation. Along with inhibition of translational initiation the translational elongation is inhibited.

The cap independent translational mechanisms of HIF and other proteins involved in hypoxic response (VEGF, EPO, etc.) are via – 1) RNA- binding proteins (RBPs) and 2) non-coding RNAs (ncRNAs). RBPs that regulate translation are PTB, HuR, hnRNPA18, and cytoplasmic polyadenylation-element binding protein (CPEB). HuR usually binds to AU or U rich mRNA sequences in their untranslated regions (UTRs) [85]. HuR binds to the 5’ UTR of the HIF-1α mRNA promoting its translation. The same phenomenon is observed even in cells treated with CoCl₂ (Upon CoCl₂ exposure cells experience hypoxia) [86]. Another RBP – PTB binds to a poly pyrimidine rich sequence preferably to a cytosine rich sequence present in the 5’UTR of the HIF-1α mRNA. The role of PTB has been suggested in the formation of a RNA loops. These loops form the Internal Ribosome Entry Sites (IRESs), recruiting the ribosome [87, 88]. However the role of PTB in HIF-1α translation via IRES is debatable. It has been shown that PTB binds to the 3’UTR of HIF-1α and stabilizes its translation independent of IRES [86]. They have demonstrated the synergistic effect of HuR binding at a different site thereby promoting HIF-1α translation [86, 89].

O₂ dependent posttranslational regulation of HIF-1α protein

Oxygen dependent regulation of HIF-1α plays a fine balance between up regulating or down regulating the protein levels in the cell. HIF-1α undergoes a plethora of posttranslational modifications that modulate its degradation.
1) Hydroxylation

As discussed earlier the main oxygen-dependent regulation of HIF-1α is hydroxylation carried out by PHDs (PHD1, PHD2, PHD3) and FIH[90]. PHD2 is the key enzyme that hydroxylates HIF-1α under normoxia on two conserved proline residues (Pro402 and Pro564) present in the ODD. [91-96]. Osteosarcoma- 9 (OS-9), to ensure proline hydroxylation of HIF-1α takes place, facilitates formation of PHD2 and HIF-1α in a stable complex [97]. Hydroxylated prolines act as a handle for the p-VHL to recognize HIF-1α’s ODD and recruit it to proteosomal degradation. If HIF-1α escapes proteosomal degradation there exists another layer of regulation i.e. asparagine hydroxylation, to prevent activation of HIF. Factor Inhibiting HIF (FIH) hydroxylates HIF-1α on the asparagine residue (Asn803) present in the C-TAD domain. This prevents recruitment of CBP/p300 co-activator complex [98] resulting in impairment of HIF transcriptional activity (Figure 3)

PHDs and FIH remain active under normoxic conditions due to the availability of their cofactors-oxygen and Fe2+ for their activity. Under hypoxia due to lowered cellular oxygen levels as well as the oxidization of Fe2+ into Fe3+ by the reactive oxygen species (ROS); HIF-1α hydroxylation regulators become inactive. Hence HIF-1α escapes hydroxylation, consequent proteosomal degradation and successfully recruits CBP/p300 co-activator complex (Figure 3).

2) Acetylation

HIF-1α under normoxia is acetylated on lysine 532 that resides in the ODD domain by arrest-defective-1 (ARD1) N-acetyltransferase. This modification acts as an additional tag that facilitates p-VHL binding on HIF-1α [99]. A non-canonical
acetyltransferase-SSAT2 is shown to acetylate a modified amino acid-thialysine (S-[2-aminoethyl]-l-cysteine) [100]. In the complex HIF regulatory pathway SSAT2 binding to HIF-1α is shown to play the role of a stabilizer of p-VHL and elongin C interaction. By promoting this interaction HIF-1α is subjected to oxygen/p-VHL dependent degradation (Figure 3).

3) Ubiquitination and Deubiquitination

Recognition of the hydroxylated prolines by p-VHL acts as a signal for the E3 substrate-recognition module. E3 ubiquitin ligase complex comprises of elongin C, elongin B, cullin-2 and ring-box 1. E3 ubiquitin ligase complex directs HIF-1α to poly-ubiquitylation [101].

p-VHL-interacting deubiquitinating enzyme 2 (VDU2) also called as USP20 is a deubiquitinating enzyme. VDU2 deubiquitylates HIF-1α [102] and is itself subjected to degradation by p-VHL [103]. Therefore HIF-1α regulation via its ubiquitylation state is interplay between p-VHL and VDU2 (Figure 3).

4) SUMOylation and DESUMOylation

Under hypoxia small ubiquitin-like modifier (SUMO)-1 is induced and co-localized with HIF-1α in the nucleus [104]. A member of protein inhibitor of activated STAT (PIAS) family (i.e. PIASy) carries out SUMOylation of HIF-1α. SUMOylation promotes degradation via hydroxyproline-independent p-VHL dependent proteosomal pathway [105, 106]. The counteracting enzyme of PIASy is SENP-1, which is a nuclear SUMO protease that DESUMOylates HIF-1α under hypoxia. This promotes HIF-1α stability and activity and regulates a positive feedback loop [107] (Figure 3).
Figure 3: Posttranslational regulation of HIF-1α protein under normoxic and hypoxic conditions.
**Ocular Neovascularization**

The retina is the most metabolically active tissue of the human body because it continuously transmits neuronal signals to the visual center of the brain that are decoded for vision. To this high-energy demanding tissue essential nutrients and oxygen are supplied by retinal and choroidal vasculatures. Retinal vasculature originates from behind the optic nerve head sending intermediate and deep capillaries into the inner segment of the retina [108], [109]. The inner retina consists of three layers – (1) ganglion cell layer (GCL) (consists of ganglion and dislodged amacrine cell nuclei), (2) inner plexiform layer (IPL) (junction of synaptic complex between dendrites of amacrine and ganglion cells and axons of bipolar cells), and (3) inner nuclear layer (INL) (consists of cell bodies and nucleus of four different cell types – amacrine, bipolar, Muller glia, and horizontal cells). The next layer (*i.e.* outer plexiform layer) (OPL) consisting of rod and cone projections separates inner retina from outer retina. The outer retina consists of outer nuclear layer (ONL) (consists of photoreceptors *i.e.* rods and cones) that sits on retinal pigment epithelial (RPE) and Bruch’s membrane complex [108, 109]. Outer retina is avascular and 90% of nutrients and oxygen are supplied by choroidal circulation [110]. It is very important that the outer retinal layer remains avascular due to the tendency of the blood vessels to capture light. Therefore the choroidal blood vessels are fenestrated that allow the plasma to pool towards the RPE’s basal surface. This facilitates photoreceptors to take nutrients and oxygen *via* processes like diffusion and uptake [108, 109].

Quiescent or homeostatic state of choroidal and retinal vasculature is very essential for proper functioning of the adult human eye. The body tightly regulates homeostasis by a proper supply of nutrients, oxygen, electrolytes, maintained by a balance between pro-
angiogenic [vascular endothelial growth factor (VEGF), platelet-derived growth factor (PDGF), stromal derived growth factor-1 (SDF-1), placental growth factor (PIGF), Angiopoietin2 (Angp2), etc.] and anti-angiogenic factors [pigment epithelium-derived factor (PEDF), Angiopoietin1 (Angp1)] [111-114]. Under different pathological conditions the homeostasis is disturbed leading to ocular neovascularization (NV).

Ocular NV is one of the major causes of blindness in people of all ages around the world (WHO, 2011). The loss of vision can occur principally due to retinal or choroidal NV [109]. Retinal NV, as the name suggests, is due to NV of vasculature and is observed in diseases like diabetic retinopathy (DR), retinopathy of prematurity (ROP), and retinal vein occlusions (RVO). Choroidal NV is observed in diseases such as age related macular degeneration (AMD) and pathological myopia, which occurs due to NV of choroidal vasculature [109].

Retinal NV is observed in many retinal ischemic diseases. In case of DR, the primary insult is hyperglycemia; in ROP, it is exposure to levels of oxygen that are higher than normal for a particular stage of retinal vascular development; in RVO, it is obstruction of vascular flow which increases hydrostatic pressure and reduces perfusion. Irrespective of their different primary triggers that cause retinal NV, they share common clinical manifestations. In the initial stages of retinal NV the blood vessels are not leaky and they grow on the retinal surface. This condition is termed as intra-retinal micro vascular abnormality [109]. In the next step the blood vessels grow aberrantly and spread towards the outer retina, reaching the vitreous layer. These retinal neovascular blood vessels connect the retinal surface to the vitreous layer. This developed network lacks tight junctions and leaks
plasma, leading to vitreous degeneration, shrinkage and contraction. Contraction of the vitreous layer pulls the retinal surface along with it, causing retinal damage and detachment resulting in blindness [109].

Clinical manifestation of choroidal NV is exhibited in two forms - classic and occult. The classic form can be diagnosed easily by fluorescein angiography. In this form the RPE is elevated as a result of drusen (protein and lipid) deposition, and subretinal hemorrhage. Occult choroidal NV is observed when NV occurs in the subretinal space (RPE and Bruch’s membrane) and leads to RPE detachment most of the time. Choroidal NV is mostly observed in AMD, which is one of the leading causes of blindness in the aged population.

Molecular pathogenesis of ocular NV is the result of activation of the HIF pathway [109]. In retinal NV the HIF pathway gets activated due to retinal ischemia causing retinal hypoxia (Figure 4). On the contrary in choroidal NV the HIF pathway gets activated due to either or all of these factors oxidative stress, physical perturbation, deposition of drusen on RPE and/or Bruch’s membrane (Figure 5). Activated HIF then binds to HRE and up regulates expression of pro-angiogenic genes and their corresponding gene receptors – VEGF: VEGFR1/VEGFR2, SDF-1: CXCR4, PDGF-B: PDGF-β, Angp2: Tie2 and, PIGF: VEGFR1 [115]. A concerted interplay between these pro-angiogenic factors leads to ocular NV. To carry out this process, endothelial progenitor cells from the bone marrow are recruited by VEGF, P1GF and SDF-1. In the eye these pro-angiogenic growth factors (secreted by retinal pigment epithelial cells, astrocytes, and muller cells) bind to their corresponding receptors on vascular endothelial cells, leading to their activation [116-118]. Angpt2 is an antagonist of Tie2 receptor (predominantly present on vascular endothelial cells) and helps in vascular remodeling [21]. This interaction leads to dephosphorylation of
Tie2 that leads to destabilization of the blood vessels enabling them to be receptive to VEGF-induced sprouting of NV [119]. In the normal physiological state, Tie2 also plays a role in maintaining the endothelial cells in a quiescent state by facilitating interaction of endothelial cells with the extracellular matrix (ECM) components [120]. Angpt2 binding to Tie2 disrupts this maintenance signal making the endothelial cells more prone to VEGF induced cascade of reactions [119]. This activation is followed by disintegration of extracellular matrix membrane and differentiation of endothelial cells into tip and stalk cells. Tip cells proliferate and form filopodic structures, migrating towards VEGF gradient, and end up forming tube like structures [109, 121] (Figure 6). These structures mature into leaky blood vessels, leading to ocular NV. Finally PDGF-B plays a role in the tube maturation by recruiting pericytes [109].
Figure 4: Flowchart representing HIF mediated molecular pathogenesis of retinal neovascularization.
Figure 5: Flowchart representing HIF mediated molecular pathogenesis of choroidal neovascularization
Figure 6: Diagrammatic representation of key steps involved in the process of neovascularization.
Current Anti-VEGF Therapies

Inhibiting NV is a promising strategy for ocular diseases like DR, AMD, RVO, etc. Owing to the prime role played by VEGF in ocular NV, targeting VEGF paved the way to successful anti-VEGF therapies [122, 123]. The FDA has approved four anti-VEGF therapies for their use in ocular NV – 1) Pegaptanib (Eyetech Pharmaceuticals (New York, NY) [124], 2) Aflibercept (Regeneron Pharmaceuticals, Tarrytown, NY) [125], 3) Ranibizumab (Genentech, South San Francisco, CA) [126], and 4) Bevacizumab (Genentech) [127]. All of the anti-VEGF therapies have been shown to exhibit similar efficacies [128].

The FDA approved Pegaptanib, the first anti-VEGF therapy for AMD, in 2004. Pegaptanib is a RNA aptamer that binds to VEGF_{165} (VEGF_{165} is a splice variant of VEGFA). This is the predominant isoform involved in pathological ocular NV) thereby neutralizing its effect. In 2004 another anti-VEGF therapy (i.e. bevacizumab) was approved for its use in colon cancer. Bevacizumab is a monoclonal antibody designed to bind all isoforms of human VEGF. Owing to its promising results shown in cancer treatments ophthalmologist began its off label use for AMD. A small open label study of bevacizumab showed promising results demonstrating positive improvement in patient’s vision. It was initially thought that bevacizumab could not diffuse across the retina, and this led to development of ranibizumab. Ranibizumab is a FDA approved therapy for AMD and is currently being used for macular edema in Europe and Australia. Ranibizumab is the smaller fragment of bevacizumab that specifically has a Fab domain that binds to VEGF. Another FDA approved anti-VEGF therapy is aflibercept, also known as VEGF-trap. This is a fusion protein made of IgG1’s Fc portion and the second and third immunoglobulin domain of
VEGF receptor 1 and 2 respectively. This is known to trap the VEGF thereby impairing its cascade of reactions.

The preferred route of administration for these anti-VEGF therapies is by intravitreal injection to avoid any systemic side effects and increase bioavailability of the drug at the site of action. However, with repeated intravitreal injections there is increased risk for infections, endophthalmitis, intraocular inflammation, increased intraocular pressure and vitreous hemorrhaging [129-131]. Systemic delivery of these anti-VEGF therapies resulted in adverse side effects like arterial-thrombotic events, transient ischemic events, hypertension, cardiac disorder and nervous system disorders [132]. Also, there are multiple growth factors that are involved in manifestation of aberrant ocular neovascularization. Hence owing to the issues with the delivery and possible adverse side effects of the current anti-VEGF therapies, there is a need for developing therapeutic strategies, which can overcome delivery issues and does not cause many adverse side effects. Most importantly there is a need to block the master regulator such as HIF or the key modulators that amplify HIF signaling. This dissertation addresses the molecular mechanisms and describes identification of several inhibitors of this nature.
CHAPTER 2

**IN VITRO SCREENING FOR HIF PATHWAY INHIBITORS**

**Introduction and rationale**

Hypoxia mediated pathological neovascularization (NV) is observed in conditions like cancer, ischemic heart diseases, stroke, diabetic retinopathy, glaucoma. Hypoxia activates a central pathway (i.e. hypoxia inducible factor (HIF) pathway) that consists of a key factor- HIF. Activated HIF results in binding of HIF to hypoxia response element (HRE) present on many pro-angiogenic genes like [hypoxia response genes (HRGs)] vascular endothelial growth factor (VEGF), insulin growth factor (IGF1), fibroblast growth factor (FGF), stromal derived growth factor-1 (SDF-1), placent al growth factor (PIGF), platelet-derived growth factor (PDGF). Molecular pathogenesis of pathological NV is associated with interplay between many pro-angiogenic factors.

Recent studies suggest that transcriptional adaptation to hypoxia also involves epigenetic changes in the histone methylation [133, 134]. HIF pathway up regulates, in addition to many known proteins that facilitate metabolic adaptation to hypoxia, transcription of a number of Jumonji-C (JmjC) domain containing iron (II), 2-oxoglutarate (2OG)-dependent histone lysine demethylases (KDMs) in the hypoxic cancer cells [7]. Of these KDMs, four (JMJD1A, JMJD2B, JMJD2C and JARID1B) are direct transcriptional targets of the HIF pathway as HIF-α/β heterodimer binds to the hypoxia response element (HRE) present in the promoters of these genes [7]. JMJD1A (also known as KDM3A or JHDM2A) regulates the expression of some hypoxia-inducible pro-angiogenic genes (adrenomedullin, ADM and growth differentiation factor 15, GDF15) by decreasing the histone methylation
levels at their promoters, and knockdown of JMJD1A reduces the tumor growth [7, 135]. Consistently, we have recently reported that hypoxia also induces the expression of KDMs in retinal pigment epithelial cells [136]. Moreover, our studies showed that the expression of pro-angiogenic genes (ADM, GDF15, HMOX1, SERPE1 and SERPB8) is dependent on KDMs under hypoxic conditions and treating the cells with a general KDM inhibitor blocks their expression.

Current attempts to inhibit pathological NV in cancers and ocular diseases, mostly use inhibitors of one or two growth factors and/or signaling intermediates. This strategy has had only partial success, and most relevant clinical trials have shown modest effects thus far. The relative inefficiency of inhibitors of single targets may be due to the multifactorial and temporal nature of the angiogenic process. Thus, possible future approaches to successfully fight pathological neovascularization may rely either on blocking some master modulators, such as HIF or KDMs (which amplify the HIF signaling), or on a combination therapy inhibiting several targets simultaneously. Towards this we have screened for phytochemicals as HIF inhibitors. We have also compared the efficacy and toxicity of the candidate HIF inhibitor with the known HIF inhibitors like cardiac glycosides (digoxin) and anthracyclines (doxorubicin).
Materials and methods

Cell culture

The five different cell lines that were used in this study are ARPE-19, D407, HT29, HEK-293 and MCF7. Except for the D407 cell line, a human RPE cell line, which was generously gifted by Dr. Richard Hunt (University of South Carolina); the other cell lines were obtained from the American Type Culture Collection (ATCC, Manassas, VA). DMEM medium, supplemented with 29 mM sodium bicarbonate, 20 mM HEPES, 10% heat inactivated FBS, 1% nonessential amino acids, 100 µg/ml of streptomycin, and 100 U/ml of penicillin (media components were purchased from US Biologicals, Massachusetts, MA), was used to grow the cells. Cells were grown in a CO₂ incubator that was maintained at 37°C, 5% CO₂, and 90% relative humidity. Seeding cell density was set to 1 million cells in 25 cm² flasks. For screening studies with honokiol, D407, HT29, HEK-293, and MCF7 were used; while ARPE-19, D407, HT29, and MCF7 were used for berberine studies. Honokiol and berberine (LKT laboratories, St. Paul, MN) were dissolved in DMSO at 0.01 M concentration. Inhibition studies were carried out by adding 5 µM, 10 µM, and 20 µM of honokiol and 50 µM berberine to the culture media with ≈70% confluent cells. Another type of human retinal pigment epithelial cell lines (ARPE-19) was used to evaluate the efficacy of honokiol with known HIF inhibitors - digoxin, doxorubicin, and honokiol. All the three drugs used in this study were prepared in DMSO. The inhibition studies were performed by adding 1 µM of digoxin or 1 µM of doxorubicin or 20 µM of honokiol to the cells. In the control samples 0.1% of DMSO, corresponding to the DMSO concentration in the cells treated with highest inhibitor concentration, was added.
**Hypoxia treatment**

Cells were treated to hypoxic condition in a bactron anaerobic chamber (Sheldon Manufacturing, Cornelius, OR). An artificial gas mix of 1% O\textsubscript{2}, 5% CO\textsubscript{2}, and 94% N\textsubscript{2} was used to maintain hypoxia in the chamber. The chamber was purged in order to generate a hypoxic atmosphere, following the manufacturer’s instructions. Partial pressure of oxygen inside the chamber was confirmed to be 1% by using dissolved oxygen meter (Extech Instruments, Nashua, NH). Upon reaching \(\approx 70\%\) confluency, cells were aseptically transferred into the hypoxic chamber through the airlocks in order to maintain the hypoxic conditions inside the chamber. To perform initial screening studies with honokiol D407 cells were exposed to 24 h hypoxia as per our earlier publications [136]. All other cell lines for all other studies were exposed to 12 h of hypoxia because maximal induction of hypoxic response genes was observed at that time point. Cell lysis for RNA extraction was performed inside the hypoxia chamber in order to avoid any exposure of the cells to normoxic conditions.

**RNA extraction from cells**

Cells grown under normoxic or hypoxic conditions, in the presence or absence of honokiol, were lysed with TRI\textsuperscript{®} reagent (Molecular Research Center, Inc., Cincinnati, OH). The lysate was collected into a micro centrifuge tube and treated with chloroform for phase separation. The aqueous phase containing RNA was separated and 0.7 volumes of isopropanol (100%) was added to precipitate RNA. After two 75% ethanol washes RNA pellet was dissolved in DNase\textregistered;RNase-free water. The RNA concentration was measured using Nanodrop (Thermo Fisher Scientific, Wilmington, DE) and 6 \(\mu\)g of RNA was reverse
transcribed using M-MLV reverse transcriptase as per manufacturer instructions (Promega, Madison, WI).

Quantitative real time PCR analysis

Quantitative real time PCR (qPCR) of all the samples were performed as described earlier [136]. Briefly, 20 µl qPCR reactions were performed with 2.5 units of Taq polymerase (Bulldog Bio, Inc., Portsmouth, NH), 0.25 mM dNTP (Fisher Scientific, Hanover Park, IL), 1 × SYBR (Invitrogen) green, 80 ng cDNA and 1.5 µM of forward and reverse primers. The qPCR reactions were performed in biological triplicates and in experimental duplicates using a LightCycler® 480 qPCR instrument (Roche Diagnostics Corporation, Indianapolis, IN). Ribosomal protein L32, β-actin, and GAPDH were used as internal controls to obtain ΔCt. The ΔCt values of the treated samples were normalized with its corresponding untreated ΔCt values to give ΔΔCt. The relative fold change in the expression levels of the genes are represented by 2−ΔΔCt value. The primer sequences used for this study are listed below in Table 1.
Table 1: Sequences of primes used for qPCR analysis

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<th>S. No</th>
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<th>Primer Sequence</th>
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<td>TCAAGTGACGAGGAGGCAT</td>
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<td>JMJD2B-qPCR-F4</td>
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**LCMS analysis of honokiol**

Purity of honokiol was evaluated by LCMS analysis using an Agilent Zorbax Eclipse XDB-C18 (4.6 × 150 mm, 3.5 µm particle size) column. ABI 3200 Q-Trap mass spectrometer fitted with electro-spray ionization (ESI) source interfaced to a Shimadzu HPLC system equipped with a diode array detector (DAD) was used for LCMS analysis. A 20 min water and acetonitrile (ACN) gradient method was run at a flow rate of 0.5 ml/min with the following parameters: 0-2 min at 5% ACN, 5% to 95% ACN in the next 4 min and holding at 95% for another 4 min, then dropping to 5% ACN in the next 8 min and holding for 2 min at 5% ACN. Honokiol was detected by setting the DAD to 280 nm. The detection in the mass spectrometer was done in the positive mode using EMS full scan.

**Cytotoxicity assay**

Cytotoxicity was determined using MTS assay at different concentrations of honokiol (5 µM, 10 µM, and 20 µM) and berberine (50, 100, and 200 µM). Cells were plated at a density of 10,000 cells/well in 96-well plate. After reaching 50-60% confluency, cells were exposed to varying concentrations of drugs under normoxic or hypoxic conditions. The media was replaced with fresh 100 µl of serum free media and Cell Titer 96® Non-Radioactive cell proliferation assay kit (Promega) was employed according to the manufacturer’s protocol. After incubating for 4 h, the quantity of formazan formed (which is proportional to the number of viable cells) was measured at 490 nm using a 96-well micro titer plate reader (Spectra Fluor Plus, Tecan, Maennedorf, Switzerland).
**Evaluation of IC\textsubscript{50} values of honokiol, digoxin and doxorubicin**

Cell viability assay was performed to determine the IC\textsubscript{50} value of each HIF inhibitor on ARPE-19 cells. Briefly, for these experiments, 10,000 cells/well were plated in to 96-well plates and exposed to varying concentrations of drugs (0.48 nM-30 µM of digoxin or doxorubicin, 10-800 µM of honokiol) for 24 h. Following this, the number of viable cells was calculated using the Premixed WST-1 Cell Proliferation Reagent according to the manufacturer’s protocol (Clontech, Mountain View, CA). This kit provides a method to measure cell proliferation based on the enzymatic cleavage of the tetrazolium salt (WST-1) to a water-soluble formazan dye, which was detected by absorbance at 450 nm using a micro titer plate reader (Analyst GT, Molecular Devices, Sunnyvale, CA). The amount of formazan formed is directly proportional to the number of viable cells. The IC\textsubscript{50} values of the three compounds were calculated using the Graph Pad Prism software.
Results

Hypoxia-mediated expression of KDMs is a conserved process

Recent studies have shown that hypoxia induces the expression of a number of KDMs in different cancer and retinal pigment epithelial cells, suggesting a new layer of epigenetic transcriptional regulation of hypoxia response genes [7, 133, 134, 136]. Although, the HRE is present in the promoters of four KDMs (JMJD1A, JMJD2B, JMJD2C and JARID1B) [7], these studies have indicated that hypoxia can induce the expression of any one to all four KDMs depending on the cell lines. Therefore, we verified the expression of these KDMs and selected markers of the HIF pathway (HIF-1α, HIF-2α, HIF-β, VEGF and GLUT) under hypoxic conditions in human retinal pigment epithelial cells (D407 and ARPE-19), human colon adenocarcinoma grade II cells (HT29), human breast adenocarcinoma cells (MCF7), and human embryonic kidney cells (HEK293). The cDNA from D407, ARPE-19, HT29, MCF7, and HEK293 cells, grown under hypoxic (1% O₂) or normoxic (21% O₂) conditions, as described in the materials and methods section; were synthesized and utilized for expression analysis by qPCR. Two independent primer pairs from different regions of the same gene were employed except for a few genes where only one validated primer pair was used. The C_t values were normalized with their respective internal controls. Since a recent report has shown that the levels of expression can vary between hypoxic and normoxic conditions depending on the internal control [137], we have exploited three different internal controls (ribosomal protein L32, β-actin, and GAPDH) to normalize the C_t values. The fold changes were calculated by comparing the hypoxic samples to their normoxic counterparts. To this end, based on the three different internal controls no significant changes in the expression levels were observed in any of the four cell lines used in this study (data not
shown); therefore, subsequent results are presented based on normalization with the ribosomal protein L32.

Consistent with previous reports, we observed an up regulation of the known HIF pathway markers (GLUT and VEGF) under hypoxic conditions in all the cell lines (Figure 7, Figure 8). These experiments further demonstrated that the expression of all four JmjC domain containing iron (II), 2OG-dependent KDMs, that are direct transcriptional target of HIF [7], also peaked within the same time frame irrespective of the origin of the cell lines (Figure 9, Figure 10). Although the expression levels of KDMs varied, depending on the cell line and the duration of the hypoxia treatment (data not shown), our studies indicated that JMJ1A was one of the strongly induced KDMs under hypoxic conditions across most cell lines. JMJ1A acts as a co-activator for nuclear hormone receptors and regulates transcription by demethylating di- and mono-methylated histone-3 lysine-9 (H3K9-me2 and H3K9-me) into H3K9, at target promoters [138]. Since recent studies have indicated that the expression of a subset of hypoxia induced genes (e.g. ADM and GDF15, which play critical roles in HIF signaling and angiogenesis [139-141]) is regulated by JMJ1A [7, 136], we further investigated the expression of these two JMJ1A targets. After hypoxia treatment, significant induction was noted for these downstream targets of JMJ1A compared to normoxic conditions (Figure 9, Figure 10). These results confirm that hypoxia-mediated induction of KDMs and JMJ1A targets is a conserved process across multiple cell lines.
**Figure 7:** Relative fold change in the mRNA levels of HIF isoforms and direct HIF targets (VEGF and GLUT) in various cell lines exposed to hypoxia: D407 (Panel A), HT29 (Panel B), MCF7 (Panel C), and HEK293 (Panel D). The bars represent relative mRNA fold change ± standard error. The horizontal line represents normoxic levels.
**Figure 8:** Relative fold change of mRNA levels of HIF isoforms and direct HIF target (VEGF) in D407 (Panel A), ARPE-19 (Panel B), HT29 (Panel C), and MCF-7 cells (Panel D) when exposed to 12 h of hypoxic treatment. The bars represent relative mRNA fold change ± standard error. The horizontal line represents normoxic levels.
Figure 9: Relative fold change in the mRNA levels of HIF regulated KDMs and JMJD1A direct targets (ADM and GDF15) in various cell lines exposed to hypoxia: D407 (Panel A), HT29 (Panel B), MCF7 (Panel C), and HEK293 (Panel D). The bars represent relative mRNA fold change ± standard error. The horizontal line represents normoxic levels.
**Figure 10:** Relative fold change of mRNA levels of HIF regulated KDMs and JMJD1A direct targets (ADM and GDF15) in D407 (Panel A), ARPE-19 (Panel B), HT29 (Panel C), and MCF-7 cells (Panel D) when exposed to 12 h of hypoxic treatment. The bars represent relative mRNA fold change ± standard error. The horizontal line represents normoxic levels.
Honokiol inhibits HIF pathway

An activation of the HIF pathway leading to hypoxia-induced NV is the central cause of pathogenesis of ocular diseases and almost all solid tumors[37, 142, 143]. These studies suggest that specific inhibitors of the HIF pathway or KDMs can be used as a new therapeutic approach to manage diseases caused by hypoxia. Therefore, in order to identify inhibitors of the HIF pathway, we evaluated the effect of a number of known phytochemicals from traditional medicine. For these experiments, D407 cells were cultured in the presence or absence of phytochemicals under normoxic and hypoxic conditions for 24 h and the level of expression of HIF isoforms (HIF-1α, HIF-2α and HIF-1β) and hypoxia markers (direct HIF target genes, VEGF and GLUT) were compared. We found that honokiol, a biphenolic phytochemical extracted from Magnolia genus [144], strongly (≈50-90\% for most genes) inhibited the expression of HIF isoforms and hypoxic markers at 5-20 µM concentrations in a concentration-dependent manner (Figure 11).
**Figure 11**: Relative fold change in the mRNA levels of HIF isoforms and direct HIF targets (VEGF and GLUT) in D407 cells when exposed to hypoxic treatment in the presence of varying concentrations of honokiol (0-20 µM). All the bars represent relative mRNA fold change ± standard error. Solid, beveled, grey, and checked bars represent 0, 5, 10, and 20 µM honokiol treatments, respectively. The horizontal line represents normoxic levels. Student t-test assuming equal variance (one tailed) was performed. All treatments are significant when compared to the control at P<0.01 represented by *.
Since commercially available honokiol is generally purified from seed cones and/or bark of the *Magnolia* tree, we evaluated its purity using a LCMS method. This study showed a single LC peak at 10.85 min and its MS peak at 10.96 min with a mass of 266.2 amu corresponding to the molecular weight of honokiol (Error! Reference source not found.). This result demonstrates that inhibition of the HIF pathway is indeed due to honokiol and not because of any impurities present in the commercial product.
Figure 12: (A) LC-MS analysis of honokiol where bottom panel shows the HPLC chromatogram observed at 280 nm with a peak at 10.85 min. Center panel shows the XIC from MS for the same sample showing a peak at 10.96 min. Top panel shows the ions at 10.96 min. (B) The chemical structure, molecular formula, and molecular weight of honokiol.
The studies with D407 cells indicated potent inhibition of the HIF pathway even at 10 µM of pure honokiol without any noticeable cytotoxicity both under hypoxic and normoxic conditions (Figure 13). Therefore, we evaluated the effect of honokiol at this concentration on the HIF pathway in other cell lines (HT29, MCF7 and HEK293). These cell lines were cultured in the presence or absence of 10 µM of honokiol under normoxic and hypoxic conditions for 12 h, and the level of expression of HIF isoforms and hypoxic markers were compared. As with D407 cells, these experiments indicated that honokiol potently (≈40-90%) inhibited the expression of most HIF isoforms and hypoxia markers (Figure 14).
Figure 13: Panel A and panel B represent cytotoxicity analysis of D407 cells with honokiol at different concentrations (0-20 µM) under normoxia and hypoxia, respectively. All the bars represent percent cell viability ± standard error. In both the panels, solid, beveled, grey, and checked bars represent 0, 5, 10, and 20 µM honokiol treatments, respectively.
**Figure 14:** Relative fold change in the mRNA levels of HIF isoforms and direct HIF targets (VEGF and GLUT) in various cell lines exposed to hypoxia: D407 (Panel A), HT29 (Panel B), MCF7 (Panel C), and HEK293 (Panel D) in presence or absence of 10 µM honokiol. Solid black and grey bars represent relative mRNA fold change ± standard error with or without honokiol, respectively. The horizontal line represents normoxic levels. Student t-test assuming equal variance (one tailed) was performed. The treatment is significant when compared to the control at $P<0.01$ represented by * and $P<0.05$ represented by #.
**Berberine inhibits the HIF pathway**

For these experiments, D407 and ARPE-19 cells were cultured in the presence or absence of phytochemicals under both normoxic and hypoxic conditions for 12 h, and the level of expression of selected markers of the HIF pathway were compared. These experiments showed that berberine, an isoquinoline alkaloid extracted from *Berberis* genus [145, 146], strongly (~75%) inhibited the expression of VEGF at 50 µM concentration without much effect on various HIF isoforms (Figure 15).

We further evaluated the effect of berberine on the HIF pathway in the cancer cell lines (HT29 and MCF7). Both the cell lines were treated with 50 µM of berberine under normoxic and hypoxic conditions for 12 h, and the level of expression of HIF isoforms and hypoxic markers were compared. Similar to RPE cells, berberine potently (~70-90%) inhibited the expression of VEGF in both the cancer cell lines without any significant effect on the expression of various HIF isoforms (Figure 15).
Figure 15: Relative fold change of mRNA levels of HIF isoforms and direct HIF target (VEGF) in D407 (Panel A), ARPE-19 (Panel B), HT29 (Panel C), and MCF-7 cells (Panel D) when exposed to 12 h of hypoxic treatment and 50 µM berberine. All the bars represent relative mRNA fold change ± standard error. Solid black bars represent no berberine treatment and grey bars represent 50 µM berberine treatment. The horizontal line represents normoxic levels. The treatment is significant when compared to the control at P<0.01 represented by *. 
Inhibition of hypoxia-mediated expression of KDMs and pro-angiogenic genes by honokiol

Recent studies have revealed that a number of KDMs, especially those that are direct HIF targets (JMJD1A, JMJD2B, JMJD2C and JARID1B), are also overexpressed in various cancers [147-151]. In renal cell carcinomas, higher levels of JMJD1A are present in hypoxic environment and around the blood vessels, suggesting its role in tumor angiogenesis [152]. JMJD1A regulates the expression of ADM and GDF15 by directly binding at their promoters [7, 136], which play critical roles in HIF signaling and angiogenesis [139-141]. Taken together, these results further emphasize that inhibition of HIF-mediated expression of KDMs such as JMJD1A, which amplify HIF signaling, could lead to a new treatment for cancers and retinal ischemic diseases.

Therefore, the effect of honokiol on hypoxia-mediated expression of KDMs and pro-angiogenic genes was evaluated. Again for these studies we focused on KDMs that are direct transcriptional target of HIF, and ADM and GDF15, that are direct transcriptional target of JMJD1A. These experiments indicated that honokiol strongly (∼40-90% for most genes) inhibited the expression of KDMs and its target pro-angiogenic genes in D407 cells in a concentration-dependent manner (Figure 16).
Figure 16: Relative fold change in the mRNA levels of HIF regulated KDMs and JMJD1A direct targets (ADM and GDF15) in D407 cells when exposed to hypoxic treatment in the presence of varying concentrations of honokiol (0-20 µM). All the bars represent relative mRNA fold change ± standard error. Solid, beveled, grey, and checked bars represent 0, 5, 10, and 20 µM honokiol treatments, respectively. The horizontal line represents normoxic levels. Student t-test assuming equal variance (one tailed) was performed. All treatments are significant when compared to the control at P<0.01 represented by *.
Further, treating the other cell lines (HT29, MCF7 and HEK293) with 10 µM of honokiol indicated that honokiol potently inhibit (≈50-90%) the expression of most the HIF pathway intermediates and markers (Figure 17). These results confirm that honokiol-mediated inhibition of the HIF pathway is conserved across multiple cell lines.
Figure 17: Relative fold change in the mRNA levels of HIF regulated KDMs and JMJD1A direct targets (ADM and GDF15) in various cell lines exposed to hypoxia: D407 (Panel A), HT29 (Panel B), MCF7 (Panel C), and HEK293 (Panel D) in presence or absence of 10 µM honokiol. Solid black and grey bars represent relative mRNA fold change ± standard error with or without honokiol, respectively. The horizontal line represents normoxic levels. Student t-test assuming equal variance (one tailed) was performed. The treatment is significant when compared to the control at P<0.01 represented by *.
Berberine inhibits hypoxia-mediated expression of KDMs and pro-angiogenic genes

Our results demonstrate that berberine, at 50 µM concentration, significantly inhibited (≈40%) the expression of most KDMs. While, a more potent inhibition (≈70-90%) was observed in the case of the JMJD1A target genes (ADM and GDF15) in both RPE and cancer cell lines (Figure 18). Finally, since our studies demonstrated that berberine inhibited the HIF pathway, its down-stream KDM and pro-angiogenic target genes at 50 µM concentration, we evaluated the cytotoxicity of berberine (at 50, 100, and 200 µM) on D407. These studies demonstrate an insignificant cytotoxicity of berberine at various concentrations under both normoxic and hypoxic conditions (Figure 19).
**Figure 18:** Relative fold change of mRNA levels of HIF regulated KDMs and JMJD1A direct targets (ADM and GDF15) in D407 (Panel A), ARPE-19 (Panel B), HT29 (Panel C), and MCF-7 cells (Panel D) when exposed to 12 h of hypoxic treatment and 50 µM berberine. All the bars represent relative mRNA fold change ± standard error. Solid black bars represent no berberine treatment and grey bars represent 50 µM berberine treatment. The horizontal line represents normoxic levels. The treatment is significant when compared to the control at P<0.01 represented by *. 
Figure 19: Cytotoxicity studies of berberine on D407 cells at 50, 100, and 200 µM concentrations.
Evaluation of IC50 values of HIF inhibitors

We have shown that honokiol and berberine are HIF inhibitors. However we have seen an anti-HIF effect of honokiol at a concentration (5 µM) 10 times lower than berberine (50 µM). We also observed cytotoxic effect of berberine on HEK-293 cells. Hence we propose that honokiol is a potent HIF inhibitor in comparison with berberine.

Recent studies have demonstrated that a number of therapeutic agents like digoxin and doxorubicin are also HIF inhibitors [153-156]. Therefore, initially we compared the toxicity and efficacy of digoxin, doxorubicin, and honokiol as HIF inhibitors. ARPE-19 cells were used to determine the IC50 values of the three compounds [157, 158]. For these viability studies, cells were exposed to varying concentrations of the drugs (0.48 nM to 30 µM for digoxin and doxorubicin; 10 µM to 800 µM for honokiol) for 24 h. The number of viable cells was calculated using the Premixed WST-1 Cell Proliferation Reagent as described in the materials and methods. Digoxin showed IC50 value of 285.40 nM (Figure 20 Panel A), doxorubicin showed IC50 value of 402.90 nM (Figure 20 Panel B), while in the case of honokiol the IC50 value was 187.40 µM for ARPE19 cells (Figure 20 Panel C). These results suggest that honokiol is far less toxic compound compared to digoxin and doxorubicin for human retinal pigment epithelial cells, and can be used at higher concentrations than digoxin and doxorubicin. Our results corroborate well with the use of honokiol as a neuroprotective agent [159].
Figure 20: Determination of IC$_{50}$ values for HIF inhibitors. Plotting the absorbance at 450 nm vs. log$_{10}$ value of drug concentrations yielded the IC$_{50}$ of 285.40 nM, 402.90 nM, and 187.40 µM for digoxin (A), doxorubicin (B), and honokiol (C), respectively.
To evaluate the efficacy of digoxin, doxorubicin, and honokiol as HIF inhibitors, we evaluated the transcription of a number of direct HIF-dependent genes under hypoxic conditions in the presence and absence of drugs. Based on the IC$_{50}$ values and the data from earlier studies [153, 154], 1μM of digoxin and doxorubicin and 20 μM of honokiol were used to perform the comparative inhibition studies. ARPE19 cells were treated with the HIF inhibitors for 12 h at ~70% confluency under hypoxia. The RNA extraction, cDNA synthesis, and qPCR analysis were performed as described in the materials and methods section. Since we have shown the induction of a number of direct HIF targets (e.g. JMJD1A, JMJD2B, JMJD2C, JARID1B, VEGF, and GLUT1) under hypoxic conditions using human retinal pigment epithelial cell lines [136, 160], we focused on the transcription levels of these genes for comparative studies. Consistent with our previous studies, following exposure to hypoxia, we observed strong induction of all the selected direct HIF targets [e.g. JMJD1A (2.4 fold), JMJD2B (3.1 fold), JMJD2C (1.5 fold), JARID1B (1.6 fold), VEGF (3.8 fold), and GLUT1 (3.7 fold)] under hypoxia relative to normoxic conditions in ARPE19 cells (Figure 21).

However, cells treated with 1 μM of digoxin and doxorubicin, which is ~3 times their IC$_{50}$ values, showed significant inhibition of hypoxic induction of all the selected HIF targets [e.g. for digoxin JMJD1A (0.4 fold), JMJD2B (1.0 fold), JMJD2C (1.0 fold), JARID1B (1.1 fold), VEGF (0.7 fold), and GLUT1 (0.4 fold); while for doxorubicin JMJD1A (0.3 fold), JMJD2B (0.6 fold), JMJD2C (0.3 fold), JARID1B (0.7 fold), VEGF (0.3 fold), and GLUT1 (0.3 fold)] under drug treated hypoxic conditions relative to untreated hypoxic conditions in ARPE19 cells (Figure 21). At the same time, treatment of cells with 20 μM honokiol, which is ~1/9$^{th}$ of the IC$_{50}$ value for ARPE19 cells, showed significant inhibition of transcription of
selected HIF targets [e.g. \textit{JMJD1A} (0.2 fold), \textit{JMJD2B} (0.6 fold), \textit{JMJD2C} (0.4 fold), \textit{JARID1B} (0.5 fold), \textit{VEGF} (0.2 fold), and \textit{GLUT1} (0.2 fold)] in ARPE19 cells (Figure 21).

\textbf{Figure 21:} Relative fold change in the mRNA levels of indicated HIF target genes in ARPE19 cells under hypoxia in the absence or presence of 1 \textmu{}M digoxin (DG), 1 \textmu{}M doxorubicin (DX), and 20 \textmu{}M honokiol. Solid, beveled, grey, and checked bars represent relative mRNA fold change ± standard error for control, 1 \textmu{}M DG, 1 \textmu{}M DX, and 20 \textmu{}M honokiol treatments, respectively. The horizontal line represents normoxic levels.
Although treatment with all three compounds resulted in lowering the transcription levels of selected HIF targets, bringing them back to or below their normoxic levels, it is interesting to note that honokiol showed better efficacy as HIF inhibitor (note that honokiol was used at ~1/9th of the IC$_{50}$ value for ARPE19 cells, while digoxin and doxorubicin were used at ~3 times higher concentrations than their IC$_{50}$ values). Similar results with respect to transcription inhibition of HIF targets were observed at as low as 5 µM concentration of honokiol. These results suggest that honokiol has a better safety to efficacy profile as a HIF inhibitor, compared to digoxin and doxorubicin, and can be used for the treatment of various ischemic ocular diseases.
**Discussion**

Pathological activation of the HIF pathway leads to the expression of pro-angiogenic factors during the NV in ocular diseases and cancer. Little is known about the epigenetic regulations associated with HIF-mediated transcription and activation of pro-angiogenic genes. Recent studies have shown that KDMs play a key role in the amplification of HIF signaling and expression of pro-angiogenic genes. In this study, we have proved that hypoxia-mediated expression of KDMs is a conserved process across multiple cell lines. Because inhibition of the HIF pathway or KDMs can have profound therapeutic value for diseases caused by pathological NV, we screened a number of phytochemicals for their ability to inhibit this pathway. These studies identified berberine and honokiol as potent inhibitors of the HIF pathway and hypoxia-mediated expression of KDMs in a number of cancer and retinal pigment epithelial cell lines. Further, treating the cells with these compounds lead to inhibition of KDM-mediated induction of pro-angiogenic genes under hypoxic conditions. However, when we compared the inhibitory effect of both these compounds we observed that honokiol showed better anti-HIF activity at a concentration 10 times lower that berberine. We warrant that honokiol is a better HIF inhibitor than berberine. When we further compared honokiol with known HIF inhibitors, digoxin and doxorubicin, we observed its anti-HIF effect at a concentration $\sim 1/9^{th}$ of the IC$_{50}$ value for ARPE19 cells (digoxin and doxorubicin showed anti-HIF effect at 3X times higher that the IC$_{50}$ on ARPE-19). These results suggest that honokiol has a better safety to efficacy profile as a HIF inhibitor, compared to digoxin and doxorubicin, and can be used for the treatment of various ocular diseases.
In traditional herbal medicines, honokiol has been used as a therapeutic agent with anti-angiogenic, anti-anxiety, anti-stroke, anti-tumor, and neuroprotective properties [144]. A number of recent studies have recapitulated these effects in preclinical models without noticeable toxicity [161]. These findings have generated interest in bringing honokiol to the clinic as a novel therapeutic agent. However, lack of comprehensive understanding of mechanism/s of honokiol-action has hindered this process. Interestingly, in a number of conditions, such as angiogenesis, stroke, neurodegeneration, etc., where honokiol is used as a therapeutic agent, pathological activation of the HIF pathway appears to play a major role in the manifestation of the disease [143]. Therefore, our results demonstrating inhibition of the HIF pathway and hypoxia-induced expression of KDMs by honokiol provides an evidence-based scientific explanation for its therapeutic benefits. Our results warrant further clinical evaluation of honokiol for the treatment of pathological ocular neovascularization.
CHAPTER 3
EVALUATING HONOKIOL’S MECHANISM OF HIF PATHWAY INHIBITION

Introduction and rationale

HIF, the master regulator of pathological NV, is a transcription factor that plays vital roles during oxygen deficiency (i.e. hypoxia) [143]. The active HIF is a heterodimeric protein and consists of two subunits, HIF-α and HIF-β/ARNT. Transcriptional regulation by oxygen is mediated by the HIF-α isoforms. In humans, three isoforms of α-subunit (HIF-1α, HIF-2α, and HIF-3α) have been identified. HIF-1α and HIF-2α are closely related and each possesses two conserved sites of prolyl hydroxylation (Pro402, Pro564 in human HIF-1α) [93, 162]. Prolyl hydroxylation at these positions by the oxygen-dependent HIF prolyl hydroxylases (PHD1-3) allows the binding of HIF-α isoforms with the von Hippel-Lindau tumor suppressor (pVHL) followed by their proteosomal degradation under normoxia [57, 95]. HIF-1α and HIF-2α also possess a highly conserved asparagine residue (Asn803 in HIF-1α, which is hydroxylated by FIH in humans) that prevents its interaction with the transcriptional co-activator p300 under normoxia [93, 162]. Under hypoxia, PHDs become inactive, allowing HIF-α to escape prolyl hydroxylation and proteosomal degradation. Subsequently, HIF-α translocates to the nucleus, dimerizes with HIF-β and recruits several transcriptional co-activators. Thus, during hypoxia, the active HIF-α/β heterodimer binds to a core DNA sequence (G/ACGTG) in the HRE present in the promoters of target genes causing overexpression of pro-angiogenic genes – VEGF, IGF1, FGF, SDF-1, PIGF, etc. Pathological NV is manifested by an orchestrated interplay between the pro-angiogenic genes. Hence it is
conspicuous to inhibit the key player-HIF, associated with the manifestation of pathological NV, in order to treat the disease. In this regard we have screened for HIF inhibitors and came up with honokiol as a candidate molecule. Honokiol is a phytochemical isolated from Magnolia genus that has been used in traditional eastern medicine. In our preliminary studies we evaluated its inhibitory effect on (mRNA levels) selected hypoxia response genes (HRGs) that play key roles in pathological NV suggesting its potential anti-HIF pathway activity. To evaluate honokiol’s mechanism of inhibition on the HIF pathway, we looked at its effect on translation and translocation (cytoplasm to nucleus) of HIF-1α; co-localization of HIF-1α with HIF-β; and finally binding of HIF to HRE.
Materials and methods

Cell culture and exposure of cells to hypoxia

In this study human retinal pigment epithelial cell line (D407) were used. D407 cells are generous gift from Dr. Richard Hunt (University of North Carolina, Chapel Hill) and were cultured under normoxia and hypoxia as reported above. Honokiol was prepared in DMSO and 0.1% of DMSO, was added to all the control samples.

Immunoblot analysis

To perform immunoblot analysis of HIF-1α, D407 cells were exposed to different conditions normoxia, hypoxia and hypoxia with 20 μM honokiol for 12 h. Following this cells were washed with 1X PBS and harvested using ice-cold RIPA buffer containing protease inhibitor cocktail. Owing to the short t1/2 of HIF-1α protein (5-10 mins), cells exposed to hypoxia were processed inside the hypoxia chamber. Cell lysate was collected and the protein concentration was determined using Bradford assay kit (Bio-Rad, Hercules, CA). For resolving the proteins 20 μg of cell lysate was loaded onto a 10% SDS-PAGE gel and then transferred onto a PVDF nitrocellulose membrane (semidy transfer was employed-Bio Rad apparatus). The membrane was blocked with 5% BSA, washed with TBST and immunoblotting was performed using mouse anti-HIF-1α primary antibody (dilution of 1:400) (ab1, abcam, Cambridge, MA). After multiple TBST washes, peroxidase-conjugated anti-mouse IgG (1:1000) secondary antibody was added. Following TBST washes, the blot was developed by adding BM chemiluminiscence reagent (Roche, Indianapolis, IN) and the image was captured using Chemi-DocXRS (Bio-Rad).
Immunofluorescence analysis

Immunofluorescence studies were performed to evaluate the effect of honokiol on -
1) translocation of HIF-1α from cytoplasm to the nucleus and 2) HIF-1α and HIF-β co-
localization in the nucleus. D407 cells were seeded in a 12 well plate on cover slips, at a
density of 1X 10^5 cells/well and incubated overnight before exposing to normoxia, hypoxia
and hypoxia with 20 µM honokiol (5, 7, 9, 12 h). After exposing cells to different conditions
they were washed with 1X PBS and fixed with 4% paraformaldehyde and 0.5 % triton X-100
(10 mins). After incubation, the solution was aspirated and washed with 1X PBS. The cover
slips were then transferred to a fresh 12 well plate and blocked with 5% goat serum (Jackson
Laboratory, Bar Harbor, ME) in 1X PBS containing 0.1 % triton X-100 (PBST). Cells were
thoroughly washed with 1X PBS and then incubated overnight with mouse anti-HIF-1α
antibody (dilution of 1:200) (ab1, abcam) at 4 °C. Following incubation, cells were washed
thoroughly with PBST and then incubated with anti-mouse alexa flour 488 antibody (dilution
1:200) (Jackson Laboratory) for 2 h at 37 °C. DAPI (0.01 µg/mL) (USB, Clevland, OH) was
added for the last 45 mins in the above incubation. Cells were washed with PBST and the
coverslips were mounted on glass slides using fluoroshield™ mounting media (Sigma, St.
Louis, MO). Confocal images for each slide were taken from 5 different positions at 100 X
magnification using Leica TCS SP5 II (Leica Microsystems, Buffalo Grove, IL). Green
channel represents HIF-1α and blue channel represents DAPI. Image J with LOCI plugin was
used to process confocal images.

To study HIF-1α and HIF-β co-localization in the nucleus the above-discussed
procedure was followed with a variation. Cells were incubated with mouse anti-HIF-1α
antibody (dilution of 1:100) (ab1, abcam) and rabbit anti-HIF-β antibody (dilution of 1:500)
(ab2, abcam) overnight at 4 °C. Following the washes they were incubated with anti-mouse alexa flour 488 antibody (dilution 1:100) (Jackson Laboratory), anti-rabbit alexa flour 585 antibody (dilution 1:500) (Jackson Laboratory) and DAPI (USB). Confocal images were taken where blue channel corresponds to DAPI, green channel corresponds to HIF-1α and red channel corresponds to HIF-β. LOCI plugin for Image J was used to process confocal images. Further, Just another co-localization program (JACoP) plugin for ImageJ was used to evaluate the extent of co-localization of HIF-1α and HIF-β in the confocal images. Using the JACoP plugin, Coste’s automatic threshold approach program was run to evaluate Mander’s coefficients M1 and M2. M1 represents co-localizing signal from green channel (HIF-1α) with red channel (HIF-β) and M2 represents vice-versa. Two-tailed Student t-test was performed.

Chromatin immunoprecipitation (ChIP)-quantitative PCR (qPCR)

To perform ChIP, SimpleChIP® Chromatin IP Kit Agarose Beads (Cell Signaling Technology, Boston, MA) was used. D407 cells were exposed to different treatment conditions (normoxia, hypoxia and hypoxia with 20 µM honokiol) for 16 h. Cells were processed as per manufacturer’s protocol. Briefly, cells were fixed by adding 1% formaldehyde to the media and later quenched by adding glycine. Following this, cells were washed with PBS and lysed in lysis buffer. Micrococal nuclease (7.5 µL) was added to digest the chromatin into 300-900 bp DNA/protein complex. Lysate was then sonicated to break the nuclear membrane and centrifuged to obtain clarified supernatant containing the chromatin material. Chromatin (30 µg) was subjected to immunoprecipitation (IP) by incubating overnight with different antibodies [anti-HIF-1α (ab2185, abcam), histone3 (cell signaling technology)] and no antibody (negative control) at 4 °C. 2% chromatin was used as the input
DNA before addition of the antibody. ChIP-grade protein G agarose beads were added to precipitate the antibody-protein-DNA complex. Following this, the complex was reverse cross-linked using proteinase K at 65 °C to obtain IP DNA which was purified. In the IP DNA, relative abundance of DNA sequence from the VEGF promoter region was analyzed by q-PCR using LightCycler®480 qPCR instrument (Roche Diagnostics Corporation, Indianapolis, IN). The primer sequences used are 5’-AGACTCCACAGTGCATACGTG-3’ (sense) and 5’-AGTGTGTCCCTCTGACAATG-3’ (anti-sense). qPCR was performed on two biological duplicates (independent ChIP experiments) and three experimental replicates (n=6). The enriched DNA after qPCR was represented as % input. It was calculated using $100 \times 2^{-\Delta C_t}$ method where $\Delta C_t = [Ct \ IP] - [Ct \ 2\% \ Input -5.4]$. The number 5.4 is the dilution factor calculated for 2% input.
Results

Honokiol inhibits binding of HIF to the HRE

We wanted to evaluate honokiol’s mode of inhibition on the HIF pathway; hence we studied it’s effect on HIF-1α translation, translocation (cytoplasm to nucleus); co-localization of HIF-1α with HIF-β; and finally evaluated HIF binding of to HRE (transcription activity of HIF).

To evaluate the effect of honokiol on HIF-1α protein expression we performed western blot analysis. Treatment with honokiol showed no significant change in the levels of HIF-1α protein expression (Figure 22). Hence honokiol does not inhibit the expression of HIF-1α at protein level and suggests the presence of an alternate mechanism for eliciting its inhibitory effect on HIF pathway.

![Western blot analysis of cell lysate from D407 cells exposed to (A) Normoxia, (B) Hypoxia, and (C) Hypoxia treated with 20 μM honokiol.](image)

Figure 22: Western blot analysis of cell lysate from D407 cells exposed to (A) Normoxia, (B) Hypoxia, and (C) Hypoxia treated with 20 μM honokiol.

Translocation of HIF-1α into the nucleus is essential for activation of the HIF pathway. Hence we looked at the effect of honokiol on this step. We observed time dependent increase in the accumulation of HIF-1α in the nucleus under hypoxia. Images of hypoxic cells starting at 9 h showed higher punctate signal in the nucleus as compared to their normoxic counterpart. This is suggestive of HIF-1α being translocated into the nucleus
by 9 h (data not shown). Based on this, we looked at the effect of honokiol on HIF-1α translocation at 12 h (Figure 23). We found no significant change in the translocation of HIF-1α upon treatment with honokiol. This suggested that honokiol had no effect on translocation of HIF-1α into the nucleus.
Figure 23: Immunofluorochemistry analysis to evaluate translocation of HIF-1α from cytoplasm to nucleus in D407 cells. Panel A – normoxia, Panel B – hypoxia and Panel C – hypoxia with 20 µM honokiol treatment. Blue channel corresponds to DAPI stain for nucleus and green channel corresponds to stain for HIF-1 α.
After HIF-1α translocates to the nucleus it dimerizes with HIF-β to form the active HIF complex in the nucleus. Therefore we wanted to carry out co-localization studies to evaluate the effect of honokiol on formation of the active HIF complex. To visually determine co-localization in 12 h normoxia, hypoxia, and hypoxia with honokiol samples, all the three channels (blue-DAPI, green- HIF-1α and red- HIF-β) were merged in ImageJ. In normoxic nuclei blue is the dominant color with streaks of red with low levels of green (Figure 24). This indicates that under normoxia there is very little HIF-1α translocation into the nucleus resulting in no colocalization with HIF-β. Unlike the normoxic sample the nuclei of hypoxia and hypoxia treated with honokiol samples are equally dominated with red and green colors. Upon merging all three channels we see clear brownish color clearly indicating co-localization of HIF-1α and HIF-β. This suggests that honokiol does not inhibit HIF-1α and HIF-β co-localization (Figure 24). M2, Mander’s colocalization coefficient was calculated using Coste’s automatic approach for ImageJ. M2 correlates to the extent of signal from HIF-β saturated with signal from HIF-1α. The value of 1 represents complete co-localization. M2 value for hypoxia is 0.82 which is statistically significant when compared to normoxia value i.e. 0.69. When treated with honokiol the M2 value is 0.81 and is not significantly different compared to hypoxia.
**Figure 24:** Immunofluorochemistry analysis to evaluate colocalization of HIF-1α and HIF-β in D407 cells. Panel A – normoxia, Panel B – hypoxia and Panel C – hypoxia with 20 µM honokiol treatment. Blue channel corresponds to DAPI stain for nucleus; green channel corresponds to stain for HIF-1α and red channel corresponds to stain for HIF-β. All three channels were merged to determine level of co-localization. JAcoP plugin for ImageJ was used to calculate Mander’s M1 and M2 coefficients using Costes automatic approach.
Under hypoxia, activated HIF is recruited onto HRE present on VEGF promoter to transcribe the VEGF gene. To evaluate if honokiol affects binding of activated HIF to the HRE we performed ChIP with anti-HIF-1α antibody. HIF-1α occupancy on the HRE was evaluated by performing qPCR using VEGF specific primers (spanning VEGF promoter region). We observed that under hypoxia HIF-1α binding on HRE is increased 4-fold compared to normoxia (% input of HIF-1α is 0.3 % under normoxia and 1.2 % under hypoxia) (Figure 25). When cells were treated with 20 μM honokiol under hypoxia, it inhibited binding of HIF-1α to HRE. We observed a significant 5-fold reduction of HIF-1α binding to HRE (% input of HIF-1α is 0.24). Therefore we conclude that honokiol exhibits its anti-HIF activity by inhibiting binding of HIF-1α to HRE.
Figure 25: ChIP - qPCR analysis of HIF-1α IP from D407 cells exposed to normoxia, hypoxia, and hypoxia treated with 20 µM honokiol. The qPCR analysis was performed on IP DNA using primers from VEGF promoter region.
Results

Most of the retinal ischemic NV diseases share a common feature of activation of the HIF pathway as an underlying condition. HIF pathway is also associated with development of many cancers and this has lead to design of several classes of HIF inhibitors. These inhibitors could be used to block the HIF pathway at different steps i.e. HIF-α: - transcription (Aminoflavone, EZN2969, etc.) [163, 164]; translation (Digoxin, Topotecan, Resveratrol, Genistein, etc.) [165-168]; stabilization by Heat Shock Protein 90 under hypoxia (Geldanamycin, etc.) [169]; translocation to the nucleus; dimerization with the HIF-β subunit; and binding of the activated HIF to the HRE (Doxorubicin, etc.).

We have identified honokiol that is a phytochemical to be a potent HIF pathway inhibitor. It has been used extensively in oriental medicine for several years. In our studies honokiol showed concentration dependent inhibition of HRGs with no known cytotoxicity. Unlike the know HIF inhibitors- digoxin and doxorubicin, it showed anti-HIF effect (5 µM) at a concentration of ~1/36 times of the IC$_{50}$ (187.4 µM) value on retinal pigment epithelial cell line. This demonstrates a better efficacy to safety profile of honokiol compared to digoxin and doxorubicin. In the current study we identified honokiol to act on the HIF pathway by inhibiting binding of HIF to the HRE. These findings provide evidence for identification of a novel class of HIF pathway inhibitors, which elicit their effect by inhibiting binding of HIF to HREs. We believe this opens avenue for design of new inhibitors using honokiol as a scaffold.
CHAPTER 4
EVALUATING HONOKIOL’S ANTI-ANGIOGENIC ACTIVITY

Introduction and rationale

Ocular NV is one of the major causes of blindness in people of all ages around the world (WHO, 2011). The loss of vision can occur due to retinal or choroidal (sub-retinal) NV. Choroidal NV is observed in diseases such as age related macular degeneration (AMD) and pathological myopia. Retinal neovascularization (NV) is observed in patients with a number of diseases such as diabetic retinopathy, retinal vein occlusions, and retinopathy of prematurity develop due to diverse predicaments damaging retinal blood vessels.

Molecular pathogenesis of ocular NV is associated with an interplay between many pro-angiogenic factors like vascular endothelial growth factor (VEGF), insulin growth factor (IGF1), fibroblast growth factor (FGF), stromal derived growth factor-1 (SDF-1), placental growth factor (PIGF), platelet-derived growth factor (PDGF), etc. In the eye these pro-angiogenic growth factors (secreted by retinal pigment epithelial cells and muller cells) bind to the receptors on retinal endothelial cells, leading to the activation of endothelial cells. This activation is followed by disintegration of extracellular matrix membrane and differentiation of endothelial cells into tip and stalk cells. Tip cells proliferate and form filopodic structures, migrating towards VEGF gradient that end up forming into tube like structures. These structures mature into leaky blood vessels leading to ocular NV.

A number of anti-angiogenic therapies targeting VEGF (e.g. Ranibizumab, Pegaptanib, Aflibercept, Bevacizumab, etc.) have been approved for the treatment of...
neovascular diseases of eye and cancer [170]. Although specific VEGF antagonists have revolutionized the treatment of these diseases [171-174], a major improvement in the vision is observed in approximately half of the patients with age-related macular degeneration [173, 174]. Further, all anti-VEGF treatments require repeated injections of VEGF antagonists at a high cost, and yet, only offer temporary respite from vascular leakage resulting in partial clinical success. This lack of efficacy of anti-VEGF therapy in this process is possibly due to implication of the HIF pathway-mediated expression of other pro-angiogenic factors like platelet-derived growth factor-B, stromal cell-derived factor 1, erythropoietin, etc. [108]. The relative inefficiency of anti-VEGF therapy may also be due to temporal nature of the angiogenic process. Thus, possible future approaches to successfully control pathological neovascularization may rely on blocking some master modulator, such as the HIF pathway.

In this regard we have shown honokiol, which is a phytochemical, extracted from the *Magnolia* genus to be a potent inhibitor of the HIF pathway [160]. It exhibits anti-HIF effect by inhibiting binding of HIF to HRE. In the current study we wanted to evaluate honokiol’s anti-angiogenic effect. Hence we used human retinal micro vascular endothelial cells (hRMVEC) to determine honokiol’s anti-angiogenic effect in the context of retinal NV by performing various *in vitro* angiogenesis assays. We also show for the first time that administration of honokiol reduces retinal neovascularization in the OIR mouse model. Honokiol also prevents the oxygen-induced central retinal vaso-obliteration, the characteristic feature of the OIR model.
Materials and methods

Cell culture and exposure of cells to hypoxia

In this study human retinal pigment epithelial cell line (D407) and human retinal microvascular endothelial cells (hRMVEC) were used. D407 cells are generous gift from Dr. Richard Hunt (University of North Carolina, Chapel Hill) and were cultured under normoxia and hypoxia as reported above. hRMVEC were purchased from Angio-Proteomie (Boston, MA) and were cultured in ENDO-growth media (Angio-Proteomie). Different concentrations of honokiol were used to evaluate its anti-angiogenic activity. Honokiol was prepared in DMSO and 0.1% of DMSO, was added to all the control samples.

Human VEGF- Enzyme-linked immunosorbent assay (ELISA)

In order to evaluate the effect of honokiol on VEGF secreted by D407 cells, ELISA was performed using human VEGF ELISA kit (RayBiotech, Inc., Norcross GA). To perform this assay D407 cells were exposed to time-dependent hypoxia (12, 24, and 36 h) with and without 20 µM honokiol. At each time point the spent media was collected and analyzed for the secreted VEGF following the manufacturer’s protocol. In brief, this is an indirect sandwich ELISA where the plate is pre coated with anti-VEGF antibody. Standards (known concentrations of VEGF) and spent media were added to each well and incubated for 2.5 h at 25°C. Bound VEGF was sandwiched with biotinylated anti-VEGF antibody and incubated for 1 hr at 25°C. To this streptavidin bound horseradish peroxidase was added which converted the chromogenic substance 3, 3', 5, 5'-Tetramethylbenzidine (TMB) (present in the substrate solution) into a colored product. The absorbance of the colored product was measured at 450 nm, which directly correlates with the amount of VEGF present in the media.
IC$_{50}$ of honokiol on hRMVEC

hRMVEC cells were plated in a 96 well plate at a density of 10,000 cells/well with varying concentrations (0.4-180 µM) of honokiol. In the wells with 0 µM honokiol 0.5% DMSO, was added that corresponds to the amount of DMSO in the highest concentration of the drug. The cells were incubated for 24 h after which, 100 µL fresh media containing 10 µL of premixed WST-1 reagent was added to each well (according to the manufacturer’s protocol) (Clontech, Mountain View, CA). After 30 mins incubation, absorbance was measured at 450 nm using Analyst GT (Molecular Devices, Sunnyvale, CA). The absorbance values were plugged into Graph Pad Prism software to calculate the IC$_{50}$ of honokiol on hRMVEC.

hRMVEC proliferation assay

This assay was performed to study the effect of VEGF (secreted by D407 cells under hypoxia) on hRMVEC proliferation. hRMVEC were plated in a 96 well plate at a density of 3000 cells/well and incubated overnight at 37°C, 5% CO$_2$ and 95% relative humidity. After the incubation ENDO-growth media was replaced with incubation media. The incubation media is ENDO-basal media with either varying concentrations of VEGF (2, 4, 6, 8, 10, 12, 14, 16 ng/mL) or the spent media collected after exposing D407 cells to different treatment conditions for 36 hrs (normoxia, hypoxia and hypoxia treated with 20 µM honokiol). Before incubating the cells with spent media, VEGF levels were quantified using ELISA, after which the spent media was diluted 1000 times (for VEGF levels to fall in the range of 2-16 ng/mL). hRMVEC cells have a doubling time of 2-3 days hence the assay was performed for 3 days. After which the media was removed and the cells were incubated with 10 µL of the
premixed- WST-1 cell proliferation kit (Clontech). Absorbance was measured at 450 nm using Analyst GT (Molecular Devices).

* hRMVEC migration assay

Oris Cell Migration Assay – Collagen I coated kit from Platypus Technologies (Madison, WI) was used to evaluate the inhibitory effect of honokiol on VEGF-induced migration of hRMVEC. The assay was performed according to the manufacturer’s instructions. Briefly, 100 µL of suspended cells (ENDO-growth media) were loaded in each well to achieve 40,000 cells/well. The cells were allowed to adhere for 6 h at 37°C and 5% CO2. After cells were attached, stopper from each well was removed except from the wells designated as reference in which the stoppers remained until results were read (t₀). Next, the cells were washed with ENDO-growth media, followed by treatment with different conditions: - ENDO-basal media, ENDO-growth media, ENDO-growth media supplemented with 50 ng/mL VEGF and, ENDO-growth media supplemented with 50 ng/mL VEGF and different concentrations of honokiol (10 and 20 µM). Cells were incubated for 16 h at 37°C and 5% CO2. Following incubation, stoppers from reference wells were removed, washed with 1X PBS, after which they were incubated with calcein AM for 30 mins at 37°C. The solution containing calcein AM was aspirated and the cells were suspended in 1X PBS. Images were obtained using a fluorescence microscope and extent of cell migration was evaluated by comparing them to the reference wells.
In vitro angiogenesis assay

In vitro angiogenesis assay’s the ability of hRMVEC to form tubes on basement membrane, under the trigger of pro-angiogenic factors (VEGF). To carry out this assay, in vitro angiogenesis tube formation kit was used (Trevigen, Gaithersburg, MD). A 96 well plate was quoted with 50 µL of reduced growth fact basement membrane extract (RGF-BME). RGF-BME was allowed to solidify by incubating at 37°C for 1 h. hRMVEC cells were plated slowly on the membrane at a density of 30,000 cells/well. The cells were treated with different treatment conditions. Cells in VEGF containing ENDO-growth media served as the positive control. The two negative controls used in this experiment were cells incubated in ENDO-basal media and cells with 100 µM sulforaphane in ENDO-growth media. In order to evaluate the effect of honokiol on the tube formation different concentrations (2.5, 5, 10, and 20 µM) in ENDO-growth media was used. The cells were incubated for 18 hrs at 37°C in a CO₂ incubator. The media was carefully aspirated and cells were washed with 150 µL PBS. Further to each well 2 µM Calcein AM (in PBS) was added and incubated for 30 min at 37°C. After the incubation Calcein AM was removed and PBS was added. Tube formation was assayed by taking fluorescence images. Efficacy of honokiol treatment was assayed by quantitating average branch length using ImageJ plugin – Angiogenesis Analyzer.

Mouse model of oxygen-induced retinopathy

C57BL/6J mice, purchased from Jackson Laboratories (Bar Harbor, ME), were used for these experiments. All experimental procedures were approved by the University of Kansas Medical Center Institutional Animal Care and Use Committee. All mice were kept in a 12 h light-dark cycle at ambient room temperature (i.e. 19-22 °C). Mice were maintained
on a standard diet for breeding (8626 rodent diet; Harlan Laboratories, Indianapolis, IN), with chow and water available ad libitum. For the OIR model, the newborn pups at P7 along with their mother were transferred to a chamber supplied with 75±2% oxygen as described [175], under continual monitoring with a ProOx 110 oxygen controller (Biospherix, Ltd., Lacona, NY) for 120 h. On P12, the mice were returned to the room air, and were given daily intraperitoneal (IP) injection of vehicle (12.5% polyethylene glycol 400 in 1× PBS) or 10-20 mg/kg of honokiol dissolved in the vehicle.

**Whole mount fluorescent staining**

Mice were anesthetized on P17 by IP injection of ketamine (1%), xylazine (0.1%), and sodium chloride (0.9%) in a concentration of 0.1 mL/10 g mouse body weight. After induction of deep anesthesia, eyes were carefully harvested and fixed in 4% paraformaldehyde for 24 h at 4 °C. A microscope was used to dissect the cornea with a circumferential limbal incision, followed by removal of the lens, vitreous, and neural retina. Retinal cups were permeabilized overnight in 0.5% Triton X-100 and 1% BSA in 1× PBS. After washing, retinae were incubated in 10 µg/mL isoelectin GS-IB4, Alexa Flour 563 conjugate (Life technologies, Grand Island, NY) in 1 mM CaCl$_2$, 2 mM NaN$_3$, 1× PBS overnight at room temperature according to established protocols [176]. After final washes with 1× PBS, retinae were flat mounted in an anti-fade medium (Southern Biotech, Birmingham, AL).

**Quantification of retinal neovascularization and confocal imaging**

Fluorescent images of the stained retinae were taken at 4× magnification using a Nikon Eclipse 80i fluorescent microscope and Nikon Elements software (Nikon, Tokyo, Japan). Adobe Photoshop CS6 software was used to photo-merge images (3-5 images/retina)
prior to analysis. Retinal neovascularization and vaso-obliteration were quantified as reported earlier [176, 177]. Both neovascularization and vaso-obliteration are represented as % of total retinal area. The total retinal area and vaso-obliteration quantifications were obtained using adobe photoshop CS6. Neovascularization was quantified using a semi-automated computer program SWIFT_NV [177], which is a set of macros run on NIH’s image J software. SWIFT_NV has a pixel cut-off value specific for each retina, based on the total retinal area, which excludes small vessel branch points from neovascularization quantification. SWIFT_NV divides the retinal image into four quadrants. During the neovascularization quantification of each quadrant, artifacts like hyper fluorescent retinal ends, hyaloid vasculature, and cellular debris were excluded from the analysis. Further, a threshold was set to highlight the neovascular tufts while excluding normal vasculature from the quantification. SWIFT_NV finally generated a stitched image highlighting the neovascular tufts on the original retinal image and gave the neovascularization area. The data is represented as means ±S.E. Students’ t-test was performed to evaluate the strength of significance. P-values less than 0.05 were considered as significant. Confocal images of the stained retinae were taken using Leica TCP SP5 II inverted microscope platform. A cross-section of leaflets from each treatment group was used to take the images. Z-stack of image was taken and the 3D projection of the stack was generated using ImageJ software.
Results

Honokiol inhibits hypoxia mediated VEGF secretion by D407 cells

HIF mediated expression of pro-angiogenic factors play a key role in retinal NV, of which overexpression of VEGF is an important factor. Currently there are many anti-VEGF therapies for retinal NV with minimal benefits for the patients. Hence we wanted to evaluate the efficacy of honokiol in inhibiting hypoxic mediated VEGF secretion from D407 cells. When D407 cells were exposed to time-dependent hypoxia (12, 24, and 36 h) we found that VEGF expression (secreted into the media) peaked at 36 h (25 µg/mL); which was quantified using ELISA (Figure 26). At the same time point, D407 cells treated with 20 µM honokiol showed up to 50 % reduction of VEGF levels (12.5 µg/mL) (Figure 26). This suggests that honokiol treatment can lower VEGF levels via inhibiting HIF pathway thereby acting as an effective anti-VEGF agent.

Figure 26: Evaluation of time dependent secretion of VEGF from D407 cells exposed to normoxia, hypoxia, and hypoxia treated with 20 µM honokiol-using ELISA.
**IC$_{50}$ of honokiol on retinal endothelial cells**

We wanted to evaluate the IC$_{50}$ of honokiol on retinal endothelial cells to determine the safe dosage range. To perform the study we used hRMVEC and were exposed to varying concentrations of the drug (0.4 - 180 µM). We found that IC$_{50}$ of honokiol on hRMVEC was at 57 µM (Figure 21). Hence we performed all of our in vitro anti-angiogenic assays between 2.5 - 20 µM.

![Graph showing dose-dependent decrease of cell viability](image)

**Figure 20:** hRMVEC cells were treated with increasing concentrations of honokiol for 24 h. Dose dependent decrease of cell viability yielded an IC$_{50}$ value of 57 µM.
*Honokiol inhibits endothelial cell proliferation*

We wanted to evaluate anti-angiogenic potential of honokiol on the key steps in NV *i.e.* endothelial cell proliferation, migration and tube formation.

In hypoxia mediated retinal NV, retina epithelial cells and muller cells secrete VEGF. This leads to activation of retinal endothelial cells and their proliferation associated with retinal NV. To mimic this process we evaluated proliferation of hRMVEC cells in response to VEGF stimulus, which is secreted in spent media collected from D407 cells. We started by evaluating the response of hRMVEC cells to varying VEGF concentrations (2, 4, 6, 8, 10, 12, 14, 16 ng/mL) and observed a concentration dependent increase in proliferation up to 12 ng/mL of VEGF (after which saturation was observed) (Figure 27). In the next step we quantified VEGF levels in the spent media (normoxia- 6.5 µg/mL, hypoxia- 9.3 µg/mL, hypoxia treated with 20 µM honokiol- 5.8 µg/mL) by performing ELISA. hRMVEC cells were treated with 1000 times diluted spent media to achieve VEGF concentrations in the range of standard curve (normoxia- 6.5 ng/mL, hypoxia- 9.3 ng/mL, hypoxia treated with 20 µM honokiol- 5.8 ng/mL). We observed that hypoxic spent media showed increased cell proliferation (upto 30%) as compared to normoxic media. Also, there was robust reduction in cell proliferation (upto 40%) when exposed to spent media from 20 µM honokiol treated sample (Figure 27). This correlated well with the VEGF levels observed in ELISA.
Figure 27: Cell proliferation assay: A) Effect of varying concentration of VEGF on proliferation of hRMVEC cells. B) Differential proliferation of hRMVEC cells treated with spent media from D407 cells exposed to normoxia, hypoxia, and hypoxia treated with 20 µM honokiol. Error bars are represented as percent standard deviation. # represents a significance of P < 0.01
Honokiol inhibits in vitro endothelial cell migration

To study the effect of honokiol concentration on migration of hRMVEC cells we performed cells migration assay. Initially we performed the assay using basal media (devoid of pro-angiogenic factors like VEGF). We do not observe hRMVEC cells migrating to the central zone (Figure 28B). When cells were treated with complete media (containing VEGF) we observe clear hRMVEC migration to the central zone (Figure 28C) as compared to t₀ well (Figure 28A). This migration increases relatively when complete media was supplemented with 50 ng/ml VEGF (Figure 28D). This indicates VEGF’s role as a chemo-attractant in cell migration. Therefore we studied the effect of honokiol (10 and 20 µM) on hRMVEC migration in complete media supplemented with 50 ng/ml VEGF. When compared to the extent of hRMVEC migration to the central zone in VEGF treated control (Figure 28D), honokiol showed concentration dependent inhibition (Figure 28E-F). Thus, honokiol acts as a potent inhibitor of VEGF-induced cell migration at a concentration above 10 µM. This observation demanded further investigation of honokiol as an anti-angiogenic compound and we performed concentration dependent tube-formation assays to supplement the claim.
Figure 28: Cell migration assay performed using hRMVEC cells for 16 h. The cells were treated with the following conditions: A) Complete media supplemented with 50 ng/ml VEGF. This was read as the reference well ($t_0$). B) Basal media. C) Complete media. D) Complete media supplemented with 50 ng/ml VEGF. E) Complete media supplemented with 50 ng/ml VEGF and 10 µM honokiol. F) Complete media supplemented with 50 ng/ml VEGF and 20 µM honokiol.
Honokiol inhibits in vitro endothelial tube formation

To evaluate the inhibitory effect of honokiol on hRMVEC’s tube formation ability, we performed *in vitro* angiogenesis assay. We initially observed that hRMVEC when cultured on RGF-BME in the presence of ENDO-basal media with (Figure 29A) and without VEGF (20 ng/mL) (Figure 29B) did not form tube like structures indicating that tube formation requires other growth factors along with VEGF. Hence we carried out our tube formation assays in ENDO-Growth media (containing a complex mixture of growth factors) and as a negative control we added 100 μM of a known angiogenic inhibitor, sulforaphane. hRMVEC formed well-networked tube structures (Figure 29C) and sulforaphane completely inhibited these tube networks (Figure 29D). This suggests that this assay could be employed to test for honokiol’s anti-angiogenic ability. We evaluated the ability of honokiol at 10, and 20 μM to inhibit tube formation (Figure 29E-F). We notice inhibitory effect of honokiol on the formation of tubes starting at 10 μM and maximum at 20 μM. When we quantitated the average branch length and evaluate the effect of honokiol, we observe a significant reduction in average branch length starting at 10 μM (Figure 30). The average tube length in CM was 240 and when treated with 10 μM honokiol it reduced to 160. It further decreased to up to 150 with increasing honokiol concentration. Hence inhibitory effect of honokiol on hRMVEC average tube length was a concentration dependent effect.
Figure 29: Tube formation assay performed using hRMVEC cells for 18 h. The cells were treated with the following conditions: A) Basal media. B) Basal media with 20 ng/ml VEGF. C) Complete media. D) Complete media with 100 µM sulforaphane. E) Complete media with 10 µM honokiol. F) Complete media with 20 µM honokiol.
Figure 30: Analysis of average branch length of the tubes formed by hRMVEC cells using angiogenesis analyzer plugin for ImageJ software.
Honokiol inhibits pathological retinal neovascular tuft formation in oxygen-induced retinopathy mouse model

Considering both digoxin and doxorubicin are examined for the treatment of ischemic ocular retinopathies [178, 179], we evaluated the efficacy of honokiol as a treatment option for these diseases in the OIR mouse model (Figure 31). This model has been extensively used to study the regulation of angiogenic factors, vascular pathogenesis, and the efficacy of antiangiogenic compounds [178, 180]. In this model, the P7 mouse pups are placed in 75% oxygen for 5 days. Exposure of 7-day-old pups to high levels of oxygen at a critical stage of retinal vascular development inhibits retinal vessel growth and causes significant oxygen-induced central retinal vessel loss (i.e. vaso-obliteration). At P12, the mice are returned to room air and due to vaso-obliteration their retina sense relative hypoxia. This activates the HIF pathway and pathological neovascularization, which is maximal at P17 [176].
**Figure 31:** Schematic representation of experimental conditions used to evaluate the efficacy of honokiol in the OIR mouse model. Mice were placed in 75% oxygen between P7-12, and then returned to room air between P12-17. This triggers a relative hypoxic condition in the retina, initiating the pathologic growth of blood vessels and neovascular tufts. Daily IP injections of vehicle or 10-20 mg/kg of honokiol dissolved in vehicle were administered from P12-16. On P17, mice were sacrificed and eyes were enucleated. Following fixation and fluorescent staining, retinae were flat mounted for imaging studies.
For our experiments, mouse pups were divided into three separate groups; group 1: vehicle-treated hyperoxia-exposed mice (sham-treated positive control), group 2: 10 mg/kg honokiol-treated hyperoxia-exposed mice (drug-treated), and group 3: 20 mg/kg honokiol-treated hyperoxia-exposed mice (drug-treated). The P12 mice from all groups, that had undergone OIR procedure, were returned to room air and vehicle or 10-20 mg/kg of honokiol dissolved in vehicle was injected daily (from P12-P16) by IP route (Figure 31). The greatest extent of neovascularization in the mouse OIR model is observed on P17. The extent of neovascularization at P17 was measured on retinal flat mounts using a computer-aided program as described in the materials and methods and (Figure 32).
Figure 32: Quantification of retinal neovascular tufts and vaso-obliteration. In the original retinal image (Panel A), the vaso-obliteration is manually marked (Panel B). The first macro of SWIFT_NV subtracts the background fluorescence from the original retinal, and divides it into four quadrants (Panel C). For each quadrant, a user-defined threshold is set in order to highlight the neovascular tufts and leave out the normal blood vessels (Panel D). Further, using the image J’s freehand tool artifacts like hyper fluorescent retinal ends, hyaloid vasculature, and cellular debris were excluded from each quadrant (Panel E). The second macro of SWIFT_NV generates a mask of the quadrant highlighting neovascular tufts (Panel F). In the same fashion other three quadrants were processed to generate the respective masks.
(Panel G). The final macro stitches all four masks to generate a composite image (Panel H). SWIFT_NV uses this image to quantify the total area of neovascular tufts. Finally, SWIFT_NV overlays the neovascular tufts on the original retinal image (Panel I).
In total, 55 retinae from 35 P17 mice were analyzed for the pathological retinal neovascular tuft formation. Note that some retinae were damaged during the processing, and therefore, were not included in the analysis. Further, the mice that weighed less than 6 g on P17 were also excluded from the analysis according to a previous publication [176]. The retinae from vehicle-treated mice (group 1) demonstrated that 4.38 ±0.95% of the total retinal area was covered by neovascular tufts (Table 2, Figure 33B). The neovascular tufts were evident by the thickening of the blood vessels and heightened staining of the vasculature with the fluorescent dye. However, fluorescent imaging studies of retinae from P17 mice from groups 2 and 3 demonstrated that daily IP injection of 10-20 mg/kg of honokiol starting on P12 significantly (~35%) reduced neovascular tufts, the clinical manifestations of the retinal neovascular response (Figure 33E and H). Interestingly, we didn’t observe a difference (2.73 ±0.65% with 10 mg/kg of honokiol vs. 2.71 ±0.62% with 20 mg/kg of honokiol) in the neovascular tufts formation in mice treated with 10 or 20 mg/kg of honokiol, suggesting that saturation of therapeutic concentration of honokiol in the retina was reached at 10 mg/kg OIR mice. Initial experiments with 0.2 and 2 mg/kg of honokiol did not have a significant effect on neovascularization in OIR mice (data not shown). Students’ t-test was performed between the vehicle-treated and honokiol-treated groups, which gave a p-value of <0.01, suggesting that our results are statistically highly significant. Further, since no report with toxicity studies of honokiol in very young mouse pups was found in the literature, we monitored the weight of pups daily from P12-P17. To this end, no weight difference was noted between the vehicle-treated or honokiol-treated groups (data not shown), and the P17 mice appeared normal with similar sizes (Figure 34).
<table>
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<tr>
<th>Group 1: sham-treated positive control, vehicle-treated hyperoxia-exposed mice</th>
<th>Neovascular tufts (of total retinal area) ±S.E.</th>
<th>Avascular area (of total retinal area) ±S.E.</th>
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<td></td>
<td>4.38 ±0.95%</td>
<td>2.24 ±0.42%</td>
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<td>Group 2: low dose drug-treated, i.e. 10 mg/kg honokiol-treated hyperoxia-exposed mice</td>
<td>2.73 ±0.65%</td>
<td>2.02 ±0.58%</td>
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<tr>
<td>Group 3: high dose drug-treated, i.e. 20 mg/kg honokiol-treated hyperoxia-exposed mice</td>
<td>2.71 ±0.62%</td>
<td>2.05 ±0.33%</td>
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**Table 2:** Table showing neovascular tufts and avascular areas in the mice retinae from groups 1-3.
Figure 33: Fluorescent imaging of the retinal flat mounts showing blood vasculature (left lane), neovascular tufts (middle lane), and avascular areas (right lane), from group 1, i.e. sham-treated hyperoxia-exposed mice (A-C), group 2, i.e. 10 mg/kg honokiol-treated hyperoxia-exposed mice (D-F), and group 3, i.e. 20 mg/kg honokiol-treated hyperoxia-exposed mice (G-I)
Honokiol inhibits retinal avascular area and promotes physiological revascularization in oxygen-induced retinopathy mouse model

To assess the vascular repair and physiological revascularization mechanisms after hyperoxia-induced vaso-obliteration, the avascular areas were quantified. Avascular areas appear as dark regions that were not perfused by fluorescent dye. The vehicle-treated ischemic retinae from group 2 mice exhibited large avascular areas (Figure 33C). However, the vaso-obliterated areas in honokiol-treated retinae from groups 2 and 3 were reduced by 10%, compared with the vehicle-treated ischemic retinae from group 1 on P17 (Figure 33F and G). Moreover, since the vascular morphology of honokiol treated retinae appears more normal than the vehicle-treated ischemic retinae (), we conclude that daily IP injections of honokiol promoted vascular repair and physiological revascularization. Taken together, these results demonstrate that honokiol is an effective therapeutic agent for the treatment of ischemic ocular neovascularization.
**Figure 34:** Representative image of mice on P17 from the vehicle treated (left), 10 mg/kg honokiol-treated (middle), and 20 mg/kg honokiol-treated (right) groups showing similar sizes.
Figure 35: Confocal images of flat mounted retina (A) Normoxic mice, (B) OIR mice, (C) OIR mice treated with 10 mg/Kg honokiol, (D) OIR mice treated with 20 mg/Kg honokiol.
Discussion

Ocular NV in the eye is one of the major causes of blindness in people of all ages (WHO, 2011). Worldwide, 2.5 million people experience vision loss due to diabetic retinopathy, whereas the age-related macular degeneration affects 25-30 million people. Other neovascular ocular diseases such as retinopathy of prematurity and retinal vascular occlusions are less prevalent but extremely debilitating with retinopathy of prematurity representing the major cause of blindness in children. Extensive studies have identified promising anti-VEGF agents to treat these debilitating diseases. However, a major improvement in the vision is observed in approximately half of the patients with age-related macular degeneration receiving Ranibizumab [173, 174]. Further, anti-VEGF therapies only offer temporary respite from vascular leakage at a very high cost. It is because of these factors that there remains an unmet medical need to develop a more cost-effective small molecule drug with new mechanism of action, which could inhibit pathological NV in patients with ocular diseases.

In the current study we evaluated honokiol’s potential as an anti-angiogenic therapeutic. We show here that honokiol inhibits secretion of VEGF by RPE cells under hypoxic conditions in a time dependent fashion. These lowered levels of VEGF secretion translated into reduction in cell proliferation of hRMVEC cells. Similarly, we observed concentration dependent inhibition of two key angiogenic processes (endothelial cell migration and tube formation).

We also show for the first time that daily IP injection of honokiol at P12 in an OIR mouse model significantly inhibits pathological retinal neovascular tuft formation at P17. Administration of honokiol prevents the oxygen-induced central retinal vaso-obliteration, and
promotes vascular recovery in the ischemic retina. Finally, our results demonstrate that honokiol, which in its crude form has been used for thousands of years in the traditional Japanese and Chinese medicine for other conditions, can also be used to inhibit pathological NV in patients with ocular diseases.
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VITA

Divya Teja Vavilala was born on August 8th, 1985, in Hyderabad, Andhra Pradesh, India. She completed her high school from Takshasila Public School, Hyderabad, Andhra Pradesh, India. She obtained her Bachelor of Technology in Biotechnology from Jawaharlal Nehru Technological University, Hyderabad, Andhra Pradesh, India in May 2007. She later obtained her Master of Science in Cell and Molecular Biology from University of Missouri – Kansas City, Kansas City, USA in May 2013.

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