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Honokiol inhibits pathological retinal neovascularization in oxygen-induced retinopathy mouse model

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ABSTRACT

Aberrant activation of the hypoxia inducible factor (HIF) pathway is the underlying cause of retinal neovascularization, one of the most common causes of blindness worldwide. The HIF pathway also plays critical roles during tumor angiogenesis and cancer stem cell transformation. We have recently shown that honokiol is a potent inhibitor of the HIF pathway in a number of cancer and retinal pigment epithelial cell lines. Here we evaluate the safety and efficacy of honokiol, digoxin, and doxorubicin, three recently identified HIF inhibitors from natural sources. Our studies show that honokiol has a better safety to efficacy profile as a HIF inhibitor than digoxin and doxorubicin. Further, we show for the first time that daily intraperitoneal 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, characteristic feature of the OIR model. Additionally, honokiol 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.

1. Introduction

Retinal neovascular diseases such as diabetic retinopathy, retinal vein occlusions, and retinopathy of prematurity develop due to diverse predicaments damaging retinal blood vessels. In the case of diabetic retinopathy, the underlying cause is hyperglycemia; in retinal vein occlusions, obstruction of vascular flow increases hydrostatic pressure and subsequently reduces perfusion; and in retinopathy of prematurity, exposure to high levels of oxygen at a particular stage of retinal vascular development. These primary insults damage retinal vessels, generating poorly perfused areas with retinal ischemia. Therefore, these diseases are collectively also referred to as ischemic retinopathies. Ocular ischemia is also observed during neovascular age-related macular degeneration and glaucoma. Irrespective of the different underlying reasons for ischemic retinopathies, they all ultimately lead to retinal hypoxia [1]. Hypoxia allows translocation of HIF-α subunit to the nucleus, its dimerization with the HIF-1β/ARNT subunit, and recruitment of other transcriptional co-activators. An active HIF-α/β heterodimeric transcription factor then binds to the hypoxia-response-element (HRE) present in the promoters of hypoxia response genes causing overexpression of vascular endothelial growth factor (VEGF) and other pro-angiogenic factors. The pathological neovascularization initiated by these pro-angiogenic factors lack tight junctions and hence leak plasma into surrounding tissues causing retinal detachment and severe vision loss.

A number of antiangiogenic therapies targeting VEGF (e.g. Ranibizumab, Pegaptanib, Aflibercept, Bevacizumab, etc.) have been approved for the treatment of neovascular diseases of eye and cancer [2]. Although specific VEGF antagonists have revolutionized the treatment of these diseases [3–6], a major improvement in the vision is observed in approximately half of the patients with age-related macular degeneration [5,6]. Further, all anti-VEGF treatments

Abbreviations: HIF, hypoxia inducible factor; HRE, hypoxia response element; PHD, prolyl hydroxylation domain; VHL, von Hippel–Lindau; VEGF, vascular endothelial growth factor; OIR, oxygen-induced retinopathy; P, postnatal day; IP, intraperitoneal.

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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. [7]. 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.

Thus characterization of novel HIF inhibitors can have considerable therapeutic impact on pathological neovascularization in ischemic diseases and cancers [8,9]. Recent screening of compounds that are in clinical practice identified cardiac glycosides (e.g. digoxin, proscillaridin A, and ouabain) and anthracyclines (e.g. doxorubicin and daunorubicin) as potent HIF inhibitors [10,11]. Since activation of the HIF pathway is the underlying cause of ocular neovascularization, both digoxin and doxorubicin are examined for the treatment of ischemic ocular retinopathies [12,13]. We have recently shown that honokiol, a biphenolic phytochemical extracted from the *Magnolia* genus, is also a potent inhibitor of the HIF pathway [14]. Here we evaluate the toxicity and efficacy of digoxin, doxorubicin, and honokiol as HIF inhibitors in retinal pigment epithelial cell lines. Further, we 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.

2. Materials and methods

2.1. Cell culture and exposure of cells to hypoxia

Human retinal pigment epithelial cell lines (D407 and ARPE19) were used to evaluate the efficacy of digoxin, doxorubicin, and honokiol. The D407 and ARPE19 cells were cultured as previously reported [14,15]. All the 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. Cells were exposed to hypoxic condition in a bactron anaerobic chamber as previously reported [14,15]. Cell lysis for RNA extraction was performed after exposure to hypoxia for 12 h in the hypoxic chamber to avoid any exposure of cells to normoxic conditions.

2.2. RNA extraction from cells and quantitative real-time PCR analysis

RNA was extracted from cells and the quantitative real-time PCR (qPCR) reactions were performed as described in our earlier publications [14,15]. The qPCR reactions were performed in biological and experimental duplicates. Ribosomal protein L32 was utilized as an internal control to normalize the sample to obtain ΔC_t value. $2^{-\Delta\Delta Ct}$ method was used to analyze the relative gene expression levels.

2.3. Cell viability assay

Cell viability assay was performed to determine the IC_{50} value of each HIF inhibitor. Briefly, for these experiments, 10,000 cells/well were plated into 96-well plates and exposed to varying concentrations (six replicates at each concentration) of HIF inhibitors 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_{50} values of the three compounds were calculated using the GraphPad Prism software.

2.4. 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 post-natal day 7 (P7) along with their mother were transferred to a chamber supplied with 75 ± 2% oxygen as described [16], 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.

2.5. 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 isolectin 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 [17]. After final washes with 1 × PBS, retinae were flat mounted in an anti-fade medium (Southern Biotech, Birmingham, AL).

2.6. Quantification of retinal neovascularization

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 [17,18]. Both neovascularization and vaso-obliteration are represented as % of total retinal area. Vaso-obliteration was manually measured using ImageJ software (http://rsbweb.nih.gov/ij/). 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 [18], 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