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Hypoxia-Inducible Factor-1 (HIF-1): A Potential Target for Intervention in Ocular Neovascular Diseases

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Abstract

Constant oxygen supply is essential for proper tissue development, homeostasis and function of all eukaryotic organisms. Cellular response to reduced oxygen levels is mediated by the transcriptional regulator hypoxia-inducible factor-1 (HIF-1). It is a heterodimeric complex protein consisting of an oxygen dependent subunit (HIF-1α) and a constitutively expressed nuclear subunit (HIF-1β). In normoxic conditions, *de novo* synthesized cytoplasmic HIF-1α is degraded by 26S proteasome. Under hypoxic conditions, HIF-1α is stabilized, binds with HIF-1β and activates transcription of various target genes. These genes play a key role in regulating angiogenesis, cell survival, proliferation, chemotherapy, radiation resistance, invasion, metastasis, genetic instability, immortalization, immune evasion, metabolism and stem cell maintenance. This review highlights the importance of hypoxia signaling in development and progression of various vision threatening pathologies such as diabetic retinopathy, retinopathy of prematurity, age-related macular degeneration and glaucoma. Further, various inhibitors of HIF-1 pathway that may have a viable potential in the treatment of oxygen-dependent ocular diseases are also discussed.

Keywords

Age-related macular degeneration; diabetic retinopathy; hypoxia signaling; hypoxia-inducible factor-1 (HIF-1); ocular neovascularization; retinopathy of prematurity

Hypoxia and the Discovery of HIF-1

A constant oxygen supply is essential for proper tissue development, homeostasis and function of all eukaryotic organisms. Cells require oxygen as an electron acceptor during oxidative phosphorylation for efficient ATP production. Oxidative phosphorylation produces higher energy (∼18 fold) than glycolysis [1, 2]. Oxygen serves as a major element in regulating membrane transport, intracellular signaling, expression of many genes, and cell survival [3, 4]. Hypoxia (∼1% O_2) occurs when tissue oxygenation demand exceeds the vascular supply. Response to reduced oxygen levels is mediated by the transcriptional

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regulator hypoxia-inducible factor 1 (HIF-1). HIF-1 was first discovered by its ability to induce expression of erythropoietin (EPO) in kidney and liver. Such production of EPO is inversely related to tissue oxygen concentration. In response to hypoxia, EPO is stimulated which in turn promotes red blood cell production and oxygen carrying capacity. This information led to the identification of a hypoxia response element (HRE; 5′-RCGTG-3′) in the 3′-enhancer region of EPO [5, 6].

Structure of HIF-1

A functional HIF-1 system is expressed in all metazoan species including the simplest animal *Trichoplax adhaerens* [7]. The *HIF1A* gene was mapped on 14q21-q24 human chromosome. HIF-1 is a heterodimeric complex consisting of an oxygen dependent subunit (HIF-1α) and a constitutively expressed nuclear subunit (HIF-1β) [8]. HIF-1β is also known as the aryl hydrocarbon receptor nuclear translocator (ARNT). It was first identified as structural binding component of aryl hydrocarbon receptor (AHR), which induces the transcription of Cyp1a1 metabolizing enzyme [9]. Both subunits are members of basic helix-loop-helix-PER-ARNT-SIM (bHLH-PAS) protein family. In human, *HIF1A*, *EPAS1* and *HIF3A* genes encode three different isoforms of HIF-α (HIF-1α, HIF-2α and HIF-3α), respectively [10].

Structurally, HIF-1α exhibits bHLH and PAS domains at the N-terminal. The bHLH domain and N-terminal of PAS (PAS-A) (amino acids/aa 1-166) facilitate DNA dimerization while complete bHLH and PAS domains (aa 1-390) facilitate DNA binding [11, 12]. HIF-1α also exhibits an oxygen-dependent degradation (ODD) domain, two transactivating domains (TADs) and two nuclear localizing signals (NLS). The ODD domain (aa 401-575) located within central region plays a predominant role in regulating stability of HIF-1α with respect to oxygen concentration [13]. Two TADs (N-TAD; aa 531-575 and C-TAD; 786-826) help in recruiting various coactivators required for transcription of target genes. Bridged between them is an inhibitory domain (ID; aa 576-785) capable of repressing their transcriptional activity under normoxic conditions (~20% O_2) [14, 15]. N-NLS (aa 17-30) and C-NLS (aa 718-721) promote nuclear translocation of HIF-1α. However, studies have demonstrated that nuclear import is highly dependent on C-NLS [16]. HIF-1α is ubiquitously expressed in all human tissues, while the other related protein HIF-2α is primarily expressed in lung, endothelium and carotid artery [17-19]. HIF-2α shares 48% structural identity with HIF-1α. A third protein, HIF-3α, is also expressed in many tissues including adult thymus, lung, brain, heart, and kidney. This protein lacks C-TAD. However, the N-terminus of this protein shares 57% and 53% structural homology with HIF-1α and HIF-2α, respectively [20, 21]. A splice variant of HIF-3α is the inhibitory PAS domain (IPAS) protein, primarily expressed in Purkinje cells and corneal epithelium. This variant acts like a negative regulator of HIF-1 by binding to amino terminal region of HIF-1α, preventing transactivation. Further, this protein is also induced under hypoxia in heart and lung, suggesting a negative feedback mechanism for HIF-1 activity [22, 23]. The domain organization of both the subunits (HIF-1α and HIF-1β) is depicted in Fig. 1.

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Regulation of HIF-1

Functional activity of HIF-1 is regulated by levels of oxygen dependent HIF-1α subunit. Although transcription of HIF-1α mRNA occurs at normoxic conditions, the protein is rapidly degraded *via* the ubiquitin proteasome pathway [24, 25]. HIF-1α protein has a very short half-life ($t_{1/2}$~5 minutes) and its stability is highly regulated by posttranscriptional modifications including hydroxylation, ubiquitination, acetylation, phosphorylation and nitrosation [26, 27].

Prolyl Hydroxylation

Hydroxylation of proline residues led to the identification of oxygen sensing mechanism of HIF-1α [28, 29]. It was considered a major breakthrough in delineating the signal transduction of HIF-1. Mutagenic studies substituting proline stabilized HIF-1α even under normal oxygen tension, demonstrate its importance in regulating transcriptional responses. Two proline residues (Pro/P 402 and 564) located within ODD domain are rapidly hydroxylated by 2-oxoglutarate (2-OG) dependent dioxygenases [30-32]. These dioxygenases recognize a conserved amino acid sequence 'LXXLAP', where X represents any amino acid. Human dioxygenases have been coined as prolyl hydroxylases (PHDs) or HIF- prolyl hydroxylases (HPHs) [33]. PHDs require oxygen for hydroxylation as well as ferrous ion (Fe^{2+}) and ascorbate as cofactors [34]. During hydroxylation an oxygen molecule is split so that one oxygen atom is transferred on to proline while the other reacts with 2-OG to produce succinate and CO_2 [30, 35]. Absolute requirement for Fe^{2+} ion stems from the observation that iron chelators or transition metal ions can suppress hydroxylation either by reducing the availability of Fe^{2+} or substituting Fe^{2+} at the active binding site [36, 37]. Ascorbate plays a very important role in regulating the activity of PHDs and maintaining the Fe^{2+} state of iron [35].

Molecular cloning studies have identified three isoforms of PHDs (PHD 1, 2 and 3) [38]. All the three isoforms can hydroxylate HIF-1α, with the highest activity exhibited by PHD2. The relative *in vitro* hydroxylation activity can be demonstrated as PHD2 $\gg$ PHD3 $>$ PHD1 [33, 39]. Subcellular localization of these isoforms varies. PHD1 is exclusively localized in the nucleus; while PHD2 is localized in the cytoplasm and PHD3 is found in both compartments. However, PHD2 is able to shuttle between cytoplasmic and nuclear components facilitating HIF-1α degradation in both compartments. Further studies have suggested that PHD2 and PHD3 mRNA expression is hypoxia inducible, while PHD1 mRNA expression is not altered by hypoxia [40].

Polyubiquitination

Post hydroxylation, von Hippel-Lindau protein (pVHL) binds HIF-1α. X-ray crystallographic studies have demonstrated that hydroxyproline fits accurately into a pocket in pVHL hydrophobic core and this binding is highly specific [41, 42]. Moreover, pVHL associates with elongin C and this interaction is stabilized by elongin B. Cullin-2 and Rbx1 proteins are also recruited to form the VCB-Cul2 E3 ligase complex which facilitates polyubiquitination and degradation by the 26S proteasome [43, 44]. Although pVHL-E3 ligase complex is predominantly expressed in the cytoplasm, cytoplasmic-nuclear trafficking

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of the complex facilitates HIF-1α degradation in both compartments [45, 46]. pVHL thus plays a predominant role in the degradation of HIF-1α. Loss of activity or mutation of pVHL has been implicated in the development of many disease processes due to induction of hypoxia regulated genes [47-49].

Lysine Acetylation

Jeong *et al*., have identified a key lysine residue (Lys/K 532) that plays a crucial role in determining proteasomal degradation of HIF-1α. An acetyl group of acetyl-coA is transferred onto K532, located within ODD domain, by acetyltransferase ARD1. This modification further promotes interaction of HIF-1α with pVHL, in concert with proline hydroxylation. ARD1 is present in all the human tissues and its activity is not dependent on oxygen levels. However, the transcriptional and translational levels of ARD1 are reduced under hypoxia, causing decreased acetylation [50]. Replacing lysine with arginine enhances stability of HIF-1α while increasing acetylation promoted its degradation [51, 52].

Asparagine Hydroxylation

A third hydroxylation site on asparagine 803 (Asn/N 803) was identified on C-TAD of HIF-1α. This asparagyl residue is conserved on HIF-2α isoform (N851) [53, 54]. Unlike other posttranslational modifications already discussed, asparagine hydroxylation may not affect the stabilization of HIF. Rather, it promotes HIF activity *via* modulation of TADs. Under normoxic conditions, N803 is hydroxylated by a factor inhibiting HIF-1 (FIH-1), an oxygen dependent 2-OG dioxygenase requiring Fe^{2+} and ascorbate as cofactors [55-57]. It is considered as a second oxygen sensor and is localized in cytoplasm. Transcription of FIH-1 is not dependent on oxygen concentration [40]. Hydroxylation on N803 prevents interaction of HIF-1α with its coactivators CREB binding protein (CBP)/p300 due to steric inhibition. This coactivator recruitment is essential for transactivation of HIF-1α [58, 59].

Phosphorylation

Phosphorylation of HIF-1α by mitogen-activated protein kinase (MAPK) pathway appears to play a crucial role in regulating its activity and function. HIF-1α is highly phosphorylated *in vitro* by p42/p44 and p38 kinases [60-62]. Such activation promotes transcriptional activity of HIF-1. It is hypothesized that HIF-1β exhibits preferential binding to the phosphorylated HIF-1α protein [63]. Inhibitors of p42/44 protein kinases diminished hypoxia induced transcriptions of target genes, while their stimulation accelerates their translational activity [64]. Threonine (Thr/T) at residues 796 and 844 appear to be the potential phosphorylation sites in HIF-1α and HIF-2α, respectively [65].

Transactivation and Target Genes of HIF-1

In normoxic conditions, *de novo* synthesized cytoplasmic HIF-1α is rapidly hydroxylated (P402 and P564) and acetylated (L532). Later, HIF-1α is captured by pVHL and degraded by 26S proteasome [28, 42, 44]. However, in hypoxic conditions hydroxylation is inhibited. It becomes stabilized and then translocates into nucleus *via* NLS. The protein heterodimerizes with constitutively expressed HIF-1β, binds to the pentacore DNA binding sequence, recruits coactivators and activates transcription of various target genes (Fig. 2)

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[66-68]. To date, more than hundred target genes of HIF-1 have been identified. The target genes play a key role in regulating angiogenesis, cell survival and proliferation, chemotherapy and radiation resistance, invasion and metastasis, genetic instability, immortalization, immune evasion, metabolism and stem cell maintenance [69-76]. Some important target genes have been listed in Table 1.

Angiogenesis is a complex signaling process involving multiple gene products [110]. Many of these genes are upregulated due to hypoxic insult [79, 111-114]. Hypoxia is an important regulatory factor directing angiogenic switch, with HIF-1 playing a predominant role in "flipping the switch" *via* direct transcriptional upregulation of vascular endothelial cell growth factor (VEGF). VEGF is a potent endothelial-specific mitogen. It interacts with its receptor (VEGFR) localized on endothelial cells and stimulates endothelial cell proliferation [112, 115-117]. Apart from VEGF induction, many complex mechanisms are also involved in HIF-1 mediated angiogenic control. The expression of α_{1B}-adrenergic receptor, adrenomedulin (ADM), angiopoietin 2, endothelin-1 (ET1), heme oxygenease-1 (HO-1), nitric oxide synthase, placental growth factor (PGF), platelet derived growth factor-B (PDGF-B) and stromal derived growth factor-1 (SDF-1) is regulated by hypoxia [105, 107-109, 118-122]. Also, expression of collagen prolyl hydroxylase, matrix metalloproteinases (MMPs) and plasminogen activator receptors and inhibitors (PAIs) under hypoxic control regulates matrix metabolism and vessel maturation (Fig. 3) [92, 99-100].

Role of HIF-1 in Ocular Diseases

Retina, a light sensitive tissue, forms the inner lining of posterior ocular segment and is metabolically one of the most active tissues in human body [123]. Continuous oxygen supply to retina facilitates high energy demand for sensitive and efficient transduction of images to readable neuronal signals [124, 125]. This neuronal function is executed by five different cell types including photoreceptors, bipolar cells, amacrine cells, horizontal cells and ganglion cells. Photoreceptors (cones and rods) play a vital role in phototransduction process. Cones mediate vision in bright light while rods mediate in dim light [126, 127]. Number of rods outweighs the number of cones by ~20 fold. Under dark conditions, a single rod cell requires 10^8 ATPs/second for ion homeostasis and signal transduction machinery. However under light exposure due to reduction in ion influx, energy requirement falls by 75% [128]. The energy requirement is met by oxidative phosphorylation process occurring in mitochondria, located within inner segment of photoreceptors [129]. Thus, oxygen concentration tightly controls retinal function.

In human a constant supply of oxygen is regulated *via* choroidal and retinal circulation. Since human retina is thick, these two separate and distinct systems act to facilitate diffusion. Choroidal vasculature nourishes the outer retina including retinal pigment epithelium (RPE) and photoreceptors while retinal vasculature perfuses the inner retinal layers. Choroidal circulation is highly vascularized. It is under low autoregulation and requires strong sympathetic control. Retinal circulation is relatively sparse, controlled by autoregulation and lacks sympathetic control. Arteriovenous oxygen gradient is also different between the two vasculatures [130, 131]. Further, the choroidal vessels are fenestrated while the retinal vessels lack fenestrations and express tight junctions [132].

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Both vasculatures play an important role in regulating retinal physiology. Lack of oxygen supply can lead to vision threatening pathologies such as diabetic retinopathy, retinopathy of prematurity, age-related macular degeneration and glaucoma. Despite the fact that initiating events are different, hypoxia with subsequent neovascularization is a characteristic phenomenon noticed with all these vascular diseases.

Diabetic Retinopathy

Diabetic retinopathy (DR) is a frequent secondary microvascular complication in patients with diabetes mellitus. It is one of the four major causes of visual impairment often leading to blindness [133, 134]. Almost 25-50% of diabetic patients exhibit retinopathy symptoms within the first 10-15 years and this number approaches nearly 100% within 30 years of diabetic onset [135, 136]. DR is characterized by biphasic progression with an initial non-proliferative (vaso-obliterative) phase followed by a proliferative (vaso-proliferative) phase. During initial stages, a persistent rise in blood glucose levels leads to a loss of intramural pericyte function. As a result small saccular capillary outpouchings, known as microaneurysms appear [137]. Intraretinal microvascular abnormalities, hemorrhages, edematous thickening of basement membrane, soft exudates and cotton wool spots are also observed [138-141]. Changes in vasculature and perturbations in oxygen tension lead to development of hypoxia, elevating the expression of angiogenic factors and subsequent neovascularization (proliferative phase) (Fig. 4A) [142]. The newly formed blood vessels are often fragile and permeable. Such vessels grow through the surface of retina into the vitreous and subsequent bleeding may lead to obstructed vision. Further, contraction of associated fibrovascular component may result in retinal detachment, vision loss and blindness [143-146].

The role of HIF-1 in the proliferative stage of DR has been clearly established. Mean oxygen tension is significantly lower in diabetics relative to non-diabetic patients in both lens (8.4 ± 0.7 mm Hg vs 10.7 ± 0.8 mm Hg) and vitreous cavity (5.7 ± 0.7 mm Hg vs 8.5 ± 0.6 mm Hg) [147]. Expression levels of HIF-1α and VEGF are elevated in diabetic preretinal membranes compared to non-diabetic idiopathic epiretinal membranes [148, 149]. Moreover, the production of VEGF and intercellular adhesion molecule (ICAM)-1 are diminished in a diabetic mice model lacking Hif-1α expression. It leads to much reduced vascular leakage and neovascularization in Hif-1α knockout mice relative to wild type mice [150]. These findings clearly suggest that alteration in HIF-1α pathway may be an attractive strategy for the treatment of DR.

Retinopathy of Prematurity

Retinopathy of prematurity (ROP), formerly known as retrolental fibroplasias, is the leading cause of visual impairment and blindness in children [151]. It was first described in early 1940s. This condition is associated with low gestational period, low birth weight and hyperoxia [152]. Human retinal vasculature begins to develop during 16th week of gestation and concludes at 40th week. Hence, premature infant's exhibit incompletely developed retinal vasculature and peripheral avascular zone. Oxygen supplementation (hyperoxia) is often needed in premature infants to overcome respiratory insufficiency. Such acute rise in oxygen tension can stimulate apoptosis of vascular endothelial cells and may cause vaso-

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obliteration *via* generation of reactive oxygen species (ROS) (Phase I). Further, high perinatal levels of prostaglandins (PGD_2 and PGE_2) and nitric oxide (NO) accelerates oxidative metabolism. Reduced levels of antioxidants may induce complexity in disease pathology [153-156]. In a subsequent phase, the infant's vaso-obliterated retina undergoes hypoxic/ischemic stress. It triggers a series of events such as stabilization of HIF-1α and production of various proangiogenic factors resulting in neovascularization (Phase II) (Fig. 4B). In contrast to normal developmental vasculature, this pathological vasculature displays excessive, uncontrolled and misdirected growth towards vitreous and lens. It can cause fibrous scarring, retinal detachment and blindness [157-160].

Given the regulatory role of oxygen, it is evident that HIF plays a predominant role in the development and progression of ROP. In a mouse oxygen-induced retinopathy (OIR) model, the expression levels of HIF-1α and HIF-2α proteins peak after two hours of hypoxic exposure. However, HIF-1α is stabilized in neuronal cells and inner retinal layers whereas HIF-2α was upregulated in Muller glia and astrocytes [161]. Indeed, inhibition of Hif-1α and Vegf by gene therapy in mice ischemic retinopathy model inhibited neovascular tufts and nuclei compared to control hypoxia group [162]. Hence, altering the HIF-1α pathway may be beneficial than targeting other downstream factors.

Age-Related Macular Degeneration

Age-related macular degeneration (AMD) is the leading cause of blindness in patients over the age of 65. World Health Organization (WHO) global eye disease survey has revealed that more than 50 million people are affected with AMD and atleast one-third of them are blind or severely visually impaired [163]. Development of AMD is multifactorial including aging, smoking, genetic factors, obesity, hypertension and hypercholesterolemia [164-168]. The disease is characterized by degeneration of central retina leading to disturbed fine and color vision. AMD is classified into two clinical forms, non-exudative/dry AMD and exudative/wet AMD [169]. The dry phase accounts for 85 to 90% of the total cases. It is characterized by the presence of acellular polymorphous debris, termed as drusen, between the basal lamina of RPE and inner collagenous layers of Bruch's membrane (Fig. 4C). As AMD progresses geographical atrophy of photoreceptors and central retina are noticed [170, 171]. Although wet AMD represents 10 to 15% of total cases, it accounts for approximately 90% of vision loss. In certain cases, dry AMD progresses to wet AMD characterized by choroidal neovascularization (CNV). These abnormal blood vessels penetrate Bruch's membrane; grow into RPE and neural retina. This may lead to blurred vision, retinal detachment, fibrosis and complete vision loss [172, 173].

Cell and molecular biology studies have demonstrated that hypoxia, chronic oxidative stress and inflammation play a key role in AMD [174-176]. Hypoxia may result from higher oxygen consumption, resulting from increased metabolic activity of the inflamed retina or due to poor circulation in central macula, resulting from vessel stenosis and microthrombosis [177-179]. Also, thickening of Bruch's membrane and drusen formation further stabilizes HIF [180]. ROS also causes elevation in HIF protein expression and results in increased transcriptional activity of hypoxia regulated genes [181, 182]. Such hypoxic milieu contributes to progression of exudative AMD and development of CNV. Infact,

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HIF-1α and HIF-2α expression were identified in endothelial cells and macrophages of choroidal neovascular membranes [183, 184].

Glaucoma

Glaucoma represents a multifactorial optic neuropathic disease [185]. It is classified into open and closed-angle depending on the anterior chamber angle. Open-angle glaucoma exhibits unobstructed and normal iridocorneal angle while the closed-angle glaucoma exhibits occlusion of the angle by the peripheral iris [186, 187]. Glaucoma is characterized by increased intraocular pressure, degeneration of retinal ganglion cells and axons. However, the exact role of hypoxia in the development of glaucoma is still unknown. Clinical observations have demonstrated retinal vascular abnormalities and impaired blood flow at the optic nerve head which may result in stabilization of hypoxic factors leading to retinal ganglion cell death [188, 189]. The expression of HIF-1α in control and glaucomatous human donor eyes was studied. Indeed, higher expression of the protein was noticed in retina and optic nerve head of glaucomatous eyes [190]. These findings clearly suggest the importance of hypoxia signaling mechanism in the pathogenesis of glaucoma.

Existing Anti-VEGF Therapeutics

Overproduction of VEGF plays an important role in pathogenesis of DR, ROP, AMD, neovascular glaucoma, central and branch retinal vein occlusion [191-194]. The human *VEGF-A* gene localized in chromosome 6p21.3 exhibits eight exons and four principal isoforms (121, 165, 189 and 206). The shorter isoform, $VEGF_{12}1$, is an acidic polypeptide and lacks heparin binding domain. The larger isoforms, $VEGF_{189}$ and $VEGF_{206}$, are highly basic and exhibit high affinity binding to heparin. The shorter isoform is freely diffusible while the larger isoforms are completely sequestered in the extracellular matrix. $VEGF_{165}$ exhibits intermediate properties, existing in both diffusible and bound forms [195]. The primary sources of VEGF in retina are RPE cells, Muller cells and ganglion cells. VEGF binds to two types of protein kinase activating receptors, VEGFR1 and VEGFR2. These high affinity receptors have been localized on retinal endothelial cells and pericytes [196-199]. Currently, anti-VEGF therapeutics are indicated in the treatment of ocular neovascular diseases. These include Pegaptanib sodium (Macugen; Eyetech Pharmaceuticals/Pfizer, NY), Ranibizumab (Leucentis; Genentech, CA), Bevacizumab (Avastin; Genentech, CA) and Aflibercept (VEGF Trap-Eye; Regeneron, NY).

Pegaptanib (50 kD) is the first anti-VEGF agent approved by US Food and Drug Administration (FDA) in 2001 for the treatment of exudative AMD. It is a 28-base ribonucleic acid aptamer, covalently linked to two branched polyethylene glycol (PEG-20kD) moieties. It binds to extracellular $VEGF_{165}$ with high affinity and prevents the interaction of VEGF with its receptor. Since pegaptanib binds specifically to only one isoform, it exhibits limited efficacy. Approval of this drug molecule began a new era in anti-VEGF therapy. Bevacizumab (149 kD) is a humanized recombinant full-length monoclonal antibody. It binds to all VEGF isoforms. Although not approved for specific intraocular use, bevacizumab has been indicated as an off-label therapeutic in the treatment of ocular diseases. Ranibizumab (48 kD) is the Fab fragment of the former, approved by US FDA in 2010 for treatment of macular edema and vein occlusion. Both these molecules bind all

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forms of VEGF. Compared to bevacizumab, ranibizumab demonstrates 5-20 fold greater potency due to higher affinity and lack of immunogenicity. Aflibercept (115 kD) was approved by US FDA in 2011 for treatment of AMD. It is a recombinant fusion protein consisting of the VEGF binding domains of human VEGFR1 and VEGFR2 fused to the Fc domain of human immunoglobulin-G1. It acts as a decoy receptor binding free VEGF [200-203].

Although large molecule therapeutics appear to be promising, their long term usage must be considered with caution. A recent investigation by Kurihara *et al.*, reported the deleterious effects following deletion of *Vegfa* gene in adult mice. Choriocapillaris are completely attenuated following three days of RPE-specific *Vegf* inactivation. Further, cone photoreceptors were damaged and cone dysfunction was noticed. These dramatic secondary "off-target" effects of *Vegf* antagonism were not observed when *Hif1a*, *Epas1*, and *Hif1a/Epas1* were genetically ablated. The transcriptional mutants did not exhibit any morphological, functional, or transcriptional differences relative to control adult mice. Further, deletion of transcription factors reduced pathological angiogenesis in laser photocoagulation model of CNV. These studies clearly reinforce the strategy that molecules aiming at HIF pathway may be an alternative, safer and effective mode of treatment than attenuating VEGF levels alone [204, 205].

Development of HIF-1 Inhibitors

Significant research has been conducted in recent years to identify inhibitors of HIF-1 pathway. Based on their putative mechanism of action, HIF inhibitors may modulate eithe i) HIF-1α mRNA expression, ii) HIF-1α protein translation, iii) HIF-1α protein degradation, iv) HIF-1α DNA binding activity and v) HIF-1α transcriptional activity. Examples of HIF-1 inhibitors are summarized in Table 2 while few of them are described below.

HIF-1α mRNA Expression

It has been hypothesized that transcriptional level of HIF-1α is the rate limiting factor of HIF-1 activity under hypoxic conditions [240]. Hence, inhibitors that effect HIF-1α mRNA expression can lower the rate of HIF-1 translation. Chen *et al.*, have studied the role of HIF-1α inhibition by RNA interference (RNAi) with shRNA in BALB/C mouse model of corneal neovascularization. The effect of shRNA treatment was assessed by measuring mean neovascularization score. The mean score values were as follows: normal (0), control neovascular eyes (3.59 ± 1.1), saline treated (4.05 ± 0.75), vehicle-treated (3.64 ± 1.02) and RNAi- treated (1.13 ± 0.96). HIF-1α shRNA reduced neovascularization by more than 3 fold compared to control eyes. Further, the expression of angiogenic factors (VEGF and MMPs) and inflammatory mediator (IL-1β) was also diminished. In summary, this study confirmed the role of HIF-1α transcriptional inhibition in reducing corneal neovascularization and associated inflammation [241]. A similar study with RNAi of HIF-1α was performed by Jiang *et al.*, in C57BL/6J mice of ischemic retinopathy. The researchers have counted the number of neovascular nuclei on the vitreal side of inner limiting membrane. The number of nuclei per cross section were as follows: normoxia (0.05 ± 0.29), hypoxia (41 ± 2.8), vehicle-treated (41 ± 2.6) and siRNA-treated (28 ± 2.8). The number of neovessels significantly decreased in transfected group relative to hypoxic group

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($p< 0.01$). This report clearly demonstrates the application of HIF-1α RNAi as a novel therapeutic for the treatment of neovascular eye diseases [162].

HIF-1α Protein Translation

Although the precise mechanism of HIF-1α protein translation in hypoxic conditions is not clear, several translational inhibitors have been identified. These molecules can directly inhibit translation or inhibit various signaling pathways (receptor tyrosine kinases, PI3K/AKT/mTOR and Ras-MAPK pathway). These signaling pathways play a predominant role in upregulating HIF-1α translation and thus inhibition of these growth factors can alter hypoxic regulation. Cardiac glycosides can inhibit HIF-1α protein translation and digoxin has been identified as a potent inhibitor in a cell-based reporter assay [242]. The effect of digoxin on ocular neovascularization was demonstrated by Yoshida *et al*., in C57BL/6 mice with ischemic retinopathy. Intraocular injection of digoxin lowered retinal neovascularization by almost 75% compared to saline group. Also, the area of CNV at Bruch's membrane was significantly lowered in presence of digoxin. Apart from inhibiting HIF-1α protein expression, digoxin also inhibited the expression of several angiogenic factors including VEGF, PDGF-B, SDF-1, VEGFR2, chemokine receptor (CXCR4) and Tie2 receptor in ischemic retina. This observation suggests that digoxin may offer advantages over VEGF antagonists in the treatment of neovascular diseases due to inhibition of several proangiogenic pathways. This study signifies that digoxin, a potent HIF-1α inhibitor, can possibly provide a better therapeutic intervention [243].

Genistein, a naturally occurring isoflavonoid, exhibits strong antiangiogenic activity. The underlying mechanism is hypothesized as inhibition of HIF-1α translation caused by inhibition of tyrosine kinases [213]. Wang *et al*., examined the effects of genistein on retinal neovascularization in C57BL/6 OIR mouse model. Number of vascular nuclei anterior to inner limiting membrane was quantified and data obtained was represented as: normoxia (0.76 ± 0.81), hypoxia (23.9 ± 4.4), genistein (50mg/kg) (20.9 ± 4.7), genistein (100mg/kg) (17.2 ± 4.0) and genistein (200mg/kg) (14.2 ± 3.2). The nuclei numbers were diminished by 87, 72 and 59% respectively, as the dose of genistein was increased. Further, dose-dependent reduction in HIF-1α and VEGF levels were also observed. This report suggests possible pharmacological application of genistein in ocular neovascularization [244].

HIF-1α Degradation

The molecular chaperone, heat shock protein 90 (Hsp90) is required for activity of various signaling proteins [245]. The interaction of Hsp90 with HIF-1α is required for proper conformational stability. Inhibitors of Hsp90 can promote degradation of HIF-1α *via* oxygen-independent proteasomal degradation [246]. Geldanamycin and its analogs (17-N-allylamino-17-demethoxygeldanamycin (17-AAG) and 17-dimethylaminoethylamino-17-demethoxygeldanamycin (17-DMAG/Deguelin)) may interfere with Hsp90/HIF-1α interaction by competing with the ATP binding site [247]. Kim *et al*. evaluated the potential of deguelin in the treatment of vaso-proliferative retinopathies. In an OIR mouse model, deguelin treated mice exhibited lower neovascularization as assessed by fluorescein angiography (Fig. 5). Further, the number of vascular lumens between posterior lens and anterior inner limiting membrane were estimated. Compared to control group the deguelin

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injected group showed lower number of vascular lumens (19 ± 3.4 vs 4 ± 2.1). Moreover, deguelin treatment did not alter normal retinal morphology as evident by normal retinal thickness and lack of any inflammation in vitreous, retina or choroid. This data clearly implies that modulation of HIF pathway reduces retinal neovascularization without any retinal toxicity [248].

HIF-1α DNA Binding and Transcriptional Activity

Binding of active heterodimeric HIF-1 to the consensus -RCGTG- enhancer element of target genes is another crucial step necessary for transcription of hypoxia inducible target genes. Also, the interaction of coactivator p300 with HIF-1α is another potential mechanism required for transcriptional activity of HIF-1. These molecular pathways can also be targeted in the treatment of various neovascular diseases [236, 249, 250].

Conclusions and Future Perspectives

In summary, high levels of energy is required for proper retinal function. Any perturbations in oxygenation may lead to progression of several retinal degenerative diseases including DR, ROP and AMD. Mechanistic studies of cellular and molecular components of hypoxia signaling have opened a new era in the treatment of retinopathies. Given the role of HIF-1 in the etiology of these diseases, it is evident that manipulation of this pathway at various stages can lead to more effective treatment of oxygen-dependent ocular diseases. As discussed in this article, many inhibitors have been identified and evaluated both in *in vitro* cell culture and animal models. However, many HIF inhibitors exhibit significant side effects and toxicities due to lack of specificity. Hence development of HIF specific inhibitors and further work validating the pharmacological intervention of these inhibitors in retinal diseases and their translation from bench to bedside is necessary. Nonetheless, there is significant optimism that modulation of HIF pathway can provide new treatments for ocular neovascular diseases.

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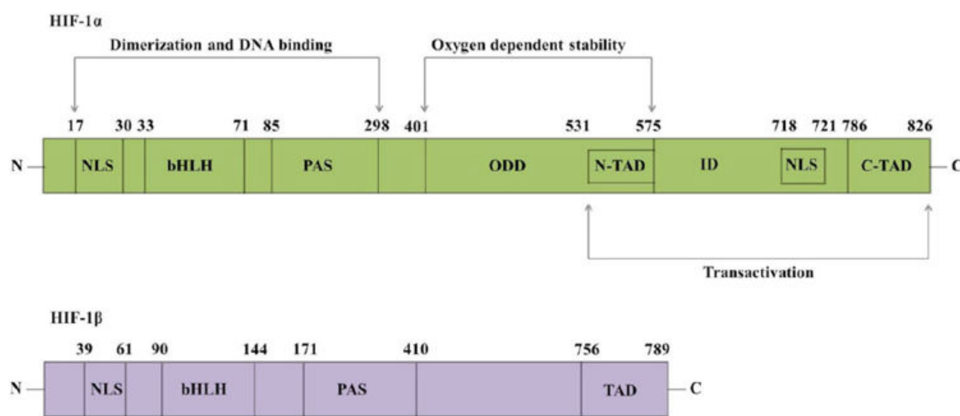

Fig. (1).

Structure of HIF-1 subunits depicting various domains. (NLS - nuclear localizing signal; bHLH - basic helix-loop-helix; PAS -PER-ARNT-SIM; ODD - oxygen-dependent degradation; TAD - transactivating domain; ID - inhibitory domain).

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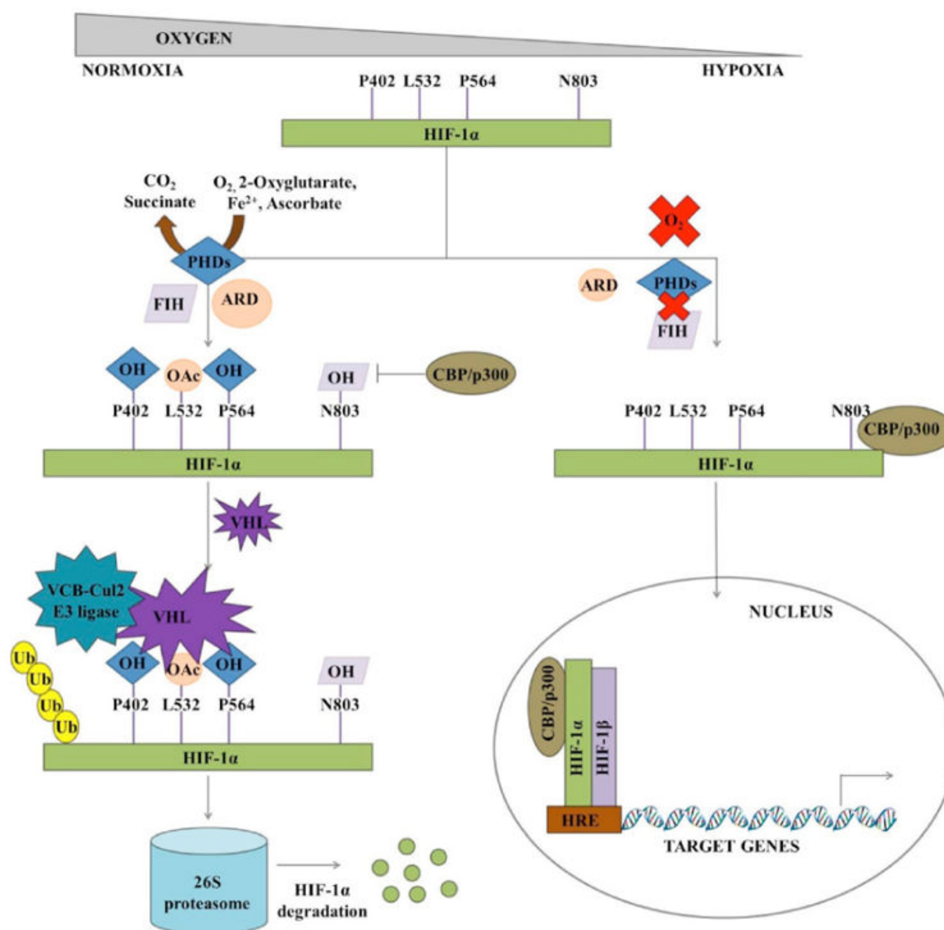

Fig. (2).

Schematic representation of oxygen dependent HIF-1 stability and transactivation. (PHD - prolyl hydroxylase; FIH - factor inhibiting HIF; ARD - acetyltransferase; CBP - CREB binding protein; VHL - von Hippel-Lindau protein; HRE - hypoxia response element).

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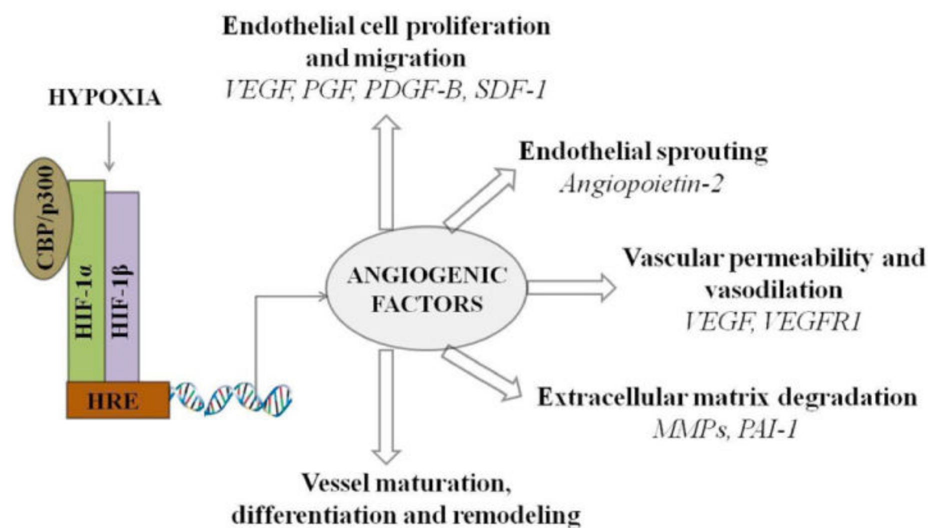

Fig. (3).

Schematic representation of angiogenic regulation by HIF-1. (VEGF - vascular endothelial cell growth factor; PGF - placental growth factor; PDGF-B - platelet derived growth factor-B; SDF-1 - stromal derived growth factor-1; VEGFR1 - VEGF receptor 1; MMPs -matrix metalloproteinases; PAI-1 - plasminogen activator inhibitor-1).

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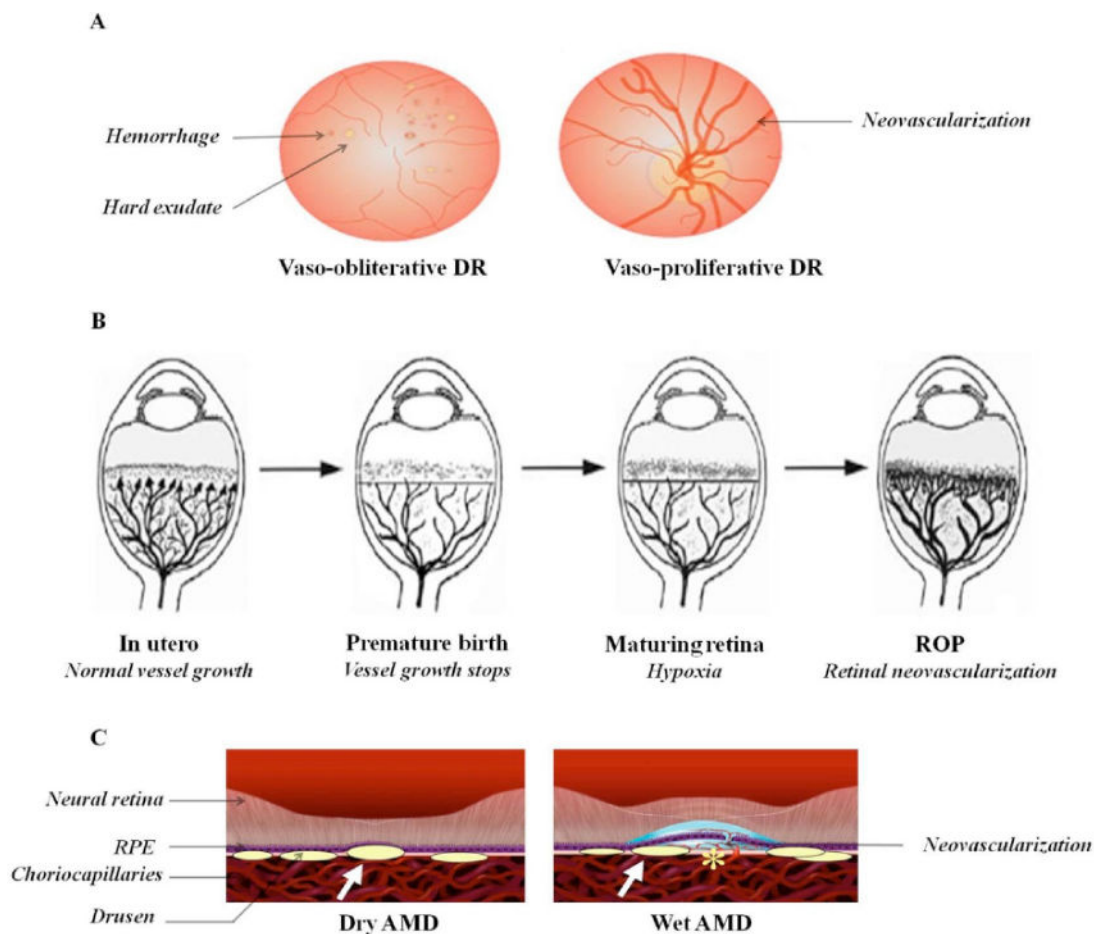

Fig. (4).

A) Stages of diabetic retinopathy (DR). B) Development of retinopathy of prematurity (ROP). Reproduced with permission from reference [152]. C) Schematic representation of dry and wet age-related macular degeneration (AMD). Reproduced with permission from reference [176].

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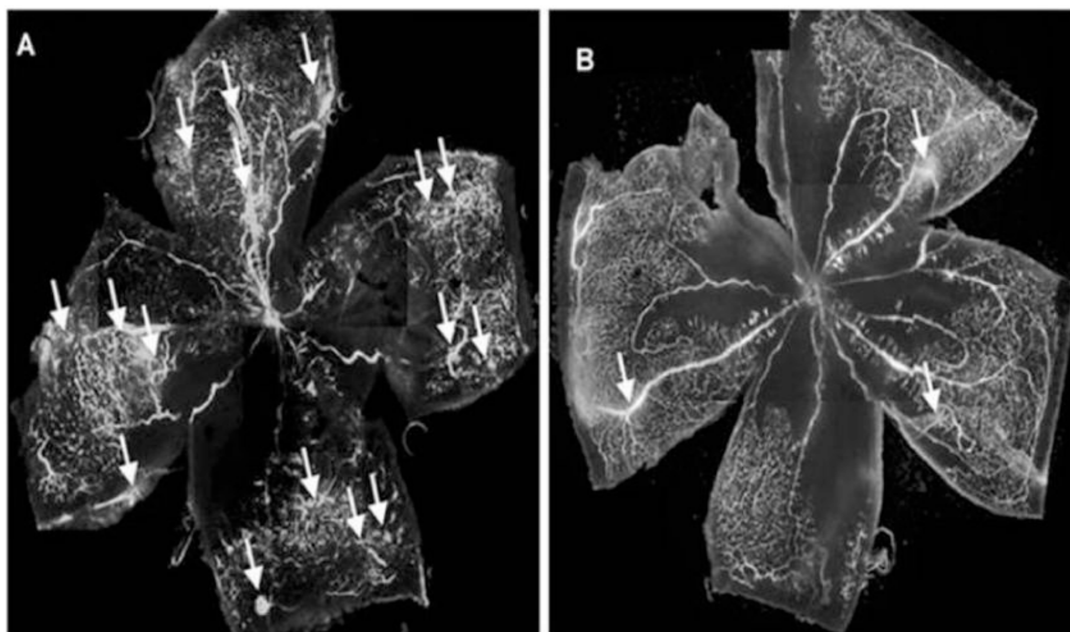

Fig. (5).

Estimation of retinal neovascularization in (A) control and (B) deguelin treated oxygen-induced retinopathy (OIR) mouse model. Reproduced with permission from reference [248].

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Table 1

Target Genes of HIF-1 Pathway

Function	Oxygen Regulated Gene	Reference
Angiogenesis	Endocrine gland derived VEGF (EG-VEGF)	[77]
	Transforming growth factor-β3 (TGF- β3)	[78]
	Vascular endothelial growth factor (VEGF)	[79]
	VEGF receptor (VEGFR1/Flt-1)	[80]
Apoptosis	Nip3	[81]
Cell proliferation and survival	Insulin-like growth factor (IGF) 2	[82]
	IGF-binding protein (IGFBP) -1	[83]
	IGFBP-2	[82]
	IGFBP-3	[82]
	Transforming growth factor- α (TGF-α)	[84]
Dedifferentiation	Inhibitor of differentiation protein-2 (ID2)	[85]
Drug resistance	Breast cancer resistance protein (BCRP)	[86]
	P-glycoprotein (P-gp/MDR1)	[87]
Energy metabolism	Leptin	[88]
Erythropoiesis	Erythropoietin (EPO)	[5]
Genetic instability	MutSalpha (MSH2 and MSH6 complex)	[89]
Glucose metabolism	Aldolase-A	[90]
	Aldolase-C	[90]
	Enolase-1	[90]
	Glucose transporter-1 (GLUT-1)	[91]
	GLUT-3	[92]
	Hexokinase-1	[90]
	Hexokinase-2	[90]
	Lactate dehydrogenase A (LDHA)	[93]
	Phosphofructokinase L (PFKL)	[93]
	Phosphoglycerate kinase 1 (PGK1)	[93]
Histone modifiers	JMJD2B	[94]
Iron metabolism	Ceruloplasmin	[95]
	Transferrin	[96]
	Transferrin receptor	[97]
Matrix metabolism	Collagen prolyl hydroxylase	[98]
	Matrix metalloproteinases (MMPs)	[99]
	Plasminogen activator inhibitor -1 (PAI-1)	[100]
Migration/Invasion	$\alpha_v\beta_3$ integrin	[101]
	Chemokine receptor (CXCR4)	[102]
Nucleotide metabolism	Adenylate kinase-3	[91]

Function	Oxygen Regulated Gene	Reference
pH regulation	Carbonic anhydrase-9	[103]
Transcriptional factor	ETS-1	[104]
Vascular tone	α_{1B}-Adrenergic receptor	[105]
	Adrenomedulin (ADM)	[106]
	Endothelin-1 (ET1)	[107]
	Heme oxygenase-1 (HO-1)	[108]
	Inducible nitric oxide synthase (iNOS)	[109]

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Table 2

Inhibitors of HIF-1 Pathway

Target Pathway / Mechanism of Action	Small Molecules	Reference
HIF-1α mRNA expression		
Transcription	Aminoflavone	[206]
	EZN-2968	[207]
	RNA interference	[208]
HIF-1α protein expression		
Translation	Digoxin	[209]
	PX-478	[210]
	Topotecan	[211]
Receptor tyrosine kinases	Erotinib	[212]
	Gefitinib	[212]
	Genistein	[213]
PI3K-AKT pathway	LY294002	[214]
	Nelfinavir	[215]
	Wortmannin	[214]
ERK-AKT pathway	Resveratrol	[216]
mTOR pathway	Everolimus	[217]
	Rapamycin	[218]
	Silibinin	[219]
	Temsirolimus	[220]
Ras-MAPK pathway	PD98059	[64]
	Sorafenib	[221]
HIF-1α protein degradation		
Hsp90 inhibitor	Apigenin	[222]
	Deguelin	[223]
	Geldanamycin	[224]
HDAC inhibitor	FK228	[225]
	SAHA	[226]
	Trichostatin A	[227]
Others		
Cyclin-dependent kinase	Flavopiridol	[228]
DNA binding	Cisplatin	[229]
	Doxorubicin	[229]
	Echinomycin	[230]
	Pyrrole - imidazole polyamide	[231]
Microtubules	2-methoxyestradiol (2ME2)	[232]
Others		
	Curcumin	[233]

Target Pathway / Mechanism of Action	Small Molecules	Reference
Mitochondria	Antimycin A1	[234]
p300 interaction	Bortezomib	[235]
	Chetomin	[236]
RNA polymerase	ECyd	[237]
Soluble guanylyl cyclase stimulator	YC-1	[238]
Thioredoxin redox system	Pleurotin	[239]

(PI3K - Phosphatidylinositide 3-Kinases; AKT - Protein Kinase B; ERK - Extracellular Signal-Regulated Kinases; mTOR - Mammalian Target of Rapamycin; Hsp - Heat Shock Protein; HDAC - Histone Deacetylase).

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