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Scholarly Review

HIF-prolyl hydroxylases as therapeutic targets in erythropoiesis and iron metabolism

Volker H. HAASE [1,2,3]

[1]Department of Medicine, Vanderbilt University Medical Center; [2]Departments of Cancer Biology and Molecular Physiology and Biophysics, Vanderbilt University School of Medicine, Nashville, Tennessee, USA; [3]Department of Veterans Affairs Hospital, Medical and Research Services, Tennessee Valley Healthcare System, Nashville, Tennessee, USA

Abstract

A classic response to systemic hypoxia is the increase in red blood cell production. This response is controlled by the prolyl hydroxylase domain/hypoxia-inducible factor (HIF) pathway, which regulates a broad spectrum of cellular functions. The discovery of this pathway as a key regulator of erythropoiesis has led to the development of small molecules that stimulate the production of endogenous erythropoietin and enhance iron metabolism. This review provides a concise overview of the cellular and molecular mechanisms that govern HIF-induced erythropoietic responses and provides an update on clinical experience with compounds that target HIF-prolyl hydroxylases for anemia therapy.

Key words: Anemia, erythropoietin, iron, hypoxia-inducible factor, prolyl hydroxylase

INTRODUCTION

A classic response to systemic hypoxia is the increased production of red blood cells (rbc). This association was first noted in the second half of the 19th century by Bert and Jourdanet,[1–3] who studied the physiologic effects of hypoxia. The search for the humoral factor that mediated this effect resulted in the purification of the glycoprotein hormone erythropoietin (EPO) in 1977, which was followed by the cloning of the *EPO* gene in 1985.[4–6] About one decade later, Semenza purified hypoxia-inducible factor (HIF) as the heterodimeric transcription factor responsible for the hypoxic induction of EPO and other oxygen-sensitive genes.[7–11] The laboratories of Ratcliffe and Kaelin then established that hydroxylation of specific proline residues within the HIF-α subunit was required for its inactivation.[12,13] This observation led to the identification of prolyl hydroxylase domain (PHD)-2, a 2-oxoglutarate (2OG)-dependent dioxygenase that utilizes molecular oxygen to carry out this hydroxylation reaction. PHD2 promotes HIF-α degradation under normoxia, whereas its inhibition results in HIF activation in most cell types.[14] Due to their seminal contributions to understanding the molecular basis of cellular oxygen sensing in metazoans, Semenza, Ratcliffe, and Kaelin received the prestigious Albert Lasker award in 2016.

Correspondence to: V. H. Haase, M.D, Division of Nephrology & Hypertension, Vanderbilt University Medical Center, C-3119A MCN, 1161 21st Avenue So., Nashville, TN 37232-2372, USA. E-mail: volker.haase@vanderbilt.edu

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THE PHD/HIF OXYGEN-SENSING PATHWAY

The PHD/HIF axis is a critically important oxygen-sensing pathway that mediates tissue adaptation to low oxygen

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environments primarily through the transcriptional regulation of gene expression. The key components of this pathway and their regulation by hypoxia are discussed in this section.

HIF transcription factors

HIFs are basic pleiotropic helix-loop-helix transcription factors that belong to the PAS (PER/aryl hydrocarbon receptor nuclear translocator [ARNT]/single minded) family of transcription factors. They consist of two subunits, an oxygen-sensitive α-subunit and a constitutively expressed β-subunit, which is often referred to as the ARNT.[15–17] The HIF-α subunit, which is continuously synthesized, is rapidly degraded in the presence of molecular oxygen. Three HIF-α-subunits have been identified, HIF-1α, HIF-2α (also known as EPAS1), and HIF-3α. Under hypoxic conditions, HIF-α is no longer degraded and translocates to the nucleus where it forms a heterodimer with HIF-β and activates gene transcription.

HIF-1 and HIF-2, the most extensively studied HIF transcription factors, facilitate oxygen delivery and cellular adaptation to hypoxia by regulating a wide spectrum of hypoxia responses such as angiogenesis, anaerobic glucose metabolism, mitochondrial biogenesis, and others.[18] The list of genes that are directly HIF-regulated is large and several hundred high stringency HIF binding sites have been identified across the genome.[19] Although HIF-1 and HIF-2 share many transcriptional targets, certain genes are not co-regulated. For example, HIF-1 regulates anaerobic glycolysis,[20] whereas EPO production and certain iron genes are HIF-2-controlled.[21–26]

HIF-prolyl hydroxylases

Under normoxic conditions, HIF-α is targeted for rapid degradation via the hydroxylation of specific proline residues (Figure 1). Hydroxylated HIF-α is tagged for proteasomal degradation by the von Hippel-Lindau (VHL) tumor suppressor protein, which functions as the substrate recognition component of an E3 ubiquitin ligase.[28,29] Under hypoxic conditions, HIF-prolyl hydroxylation is reduced, HIF-α is no longer degraded and translocates to the nucleus where it heterodimerizes with HIF-β and activates gene transcription (Figure 1). Any reduction in HIF-proline hydroxylation or impairment in VHL function decreases HIF degradation and results in increased HIF target gene expression. For example, patients with certain inactivating VHL mutations are predisposed to the development of CNS hemangioblastomas, clear cell renal cancer, and pheochromocytomas, tumors

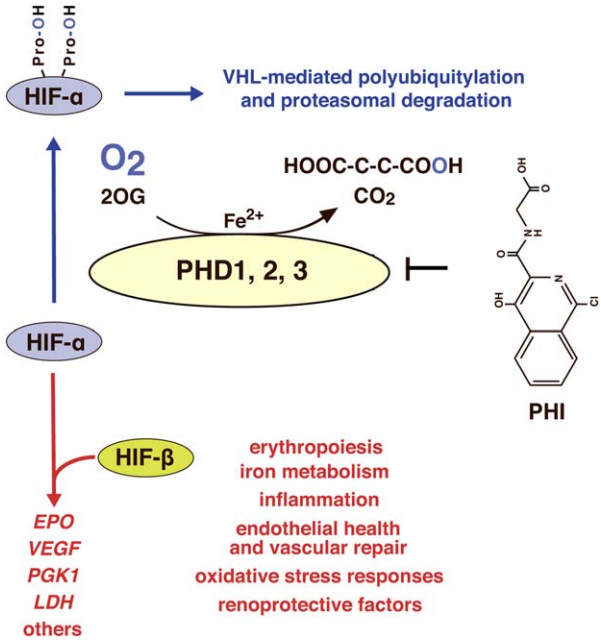

Figure 1 Schematic overview of the PHD (prolyl hydroxylase domain)/hypoxia-inducible factor (HIF) pathway. Although the oxygen-sensitive α-subunit of HIF is constitutively synthesized, it is rapidly degraded under normoxic conditions. Under hypoxia, however, cellular HIF-α levels build up and HIF-α translocates to the nucleus, where it forms a heterodimer with HIF-*β*. Proteasomal degradation of HIF-α is mediated by the pVHL-E3-ubiquitin ligase complex and requires HIF-α prolyl-4-hydroxylation by oxygen- and iron-dependent PHD dioxygenases (PHD1-3). The decarboxylation of 2-oxoglurate (2OG) produces hydroxylated HIF-α, succinate and CO_2. PHD or von Hippel-Lindau inhibition results in increased transcription of HIF-regulated genes such as *vascular endothelial growth factor* (*VEGF*), *erythropoietin* (*EPO*), *phosphoglycerate kinase 1* (*PGK1*), *lactate dehydrogenase* (*LDH*), and other genes involved in the regulation of hypoxia responses, including cellular metabolism and mitochondrial function, inflammation, vascular function, and oxidative stress and other responses. Shown is the chemical structure of a HIF-PHD inhibitor (PHI) capable of effectively stimulating the production of endogenous EPO in hemodialysis patients.[27]

which are characterized by increased HIF-regulated gene expression.[30] Furthermore, certain mutations that affect the ability of cells to effectively hydroxylate HIF-α are associated with abnormal regulation of HIF activity and result in the development of polycythemia.[31]

Oxygen-dependent hydroxylation of HIF-α is carried out by PHD1, PHD2, and PHD3, which function as oxygen sensors in the HIF pathway.[32] PHDs belong to a large family of 2OG-dependent dioxygenases with more than

60 members. Because these dioxygenases utilize molecular oxygen for hydroxylation, they link oxygen, intermediary, and amino acid metabolism to various cellular processes that include HIF regulation/hypoxia responses, collagen synthesis, epigenetic gene regulation, and fatty acid metabolism.[33]

Small molecules, such as reactive oxygen species, nitric oxide, and the Krebs cycle intermediates succinate and fumarate inhibit PHD catalytic activity leading to HIF-α stabilization and activation of HIF transcriptional programs.[32] The latter is clinically relevant in patients with fumarate hydratase deficiency who are predisposed to the development of the hereditary leiomyomatosis renal cell cancer syndrome, which is characterized by increased HIF activity in affected tissues.[34,35] Structural analogs of 2OG that block 2OG and HIF-α from accessing the PHD catalytic center and thus reversibly inhibit HIF-α hydroxylation are now under clinical investigation for renal anemia and other indications.[36] Current clinical experience with these compounds is discussed below.

THE PHD/HIF AXIS IN ERYTHROPOIESIS AND IRON METABOLISM

The PHD/HIF axis coordinates hypoxia responses across multiple cell types and tissues. In addition to renal and hepatic EPO production, HIF controls iron uptake and utilization and facilitates erythroid progenitor maturation and proliferation in the bone marrow. Key aspects of renal and hepatic EPO regulation and the mechanisms by which HIF controls iron homeostasis are discussed in this section.

Oxygen-dependent EPO production in the kidney

The kidney is the main source of EPO in adults, where it is synthesized by perivascular fibroblasts and pericytes.[36] In chronic kidney injury, renal perivascular interstitial cells and pericytes are also major cellular sources of myofibroblasts, which are interstitial cells that promote fibrosis and progression of CKD through excessive collagen production. Therefore, perivascular interstitial cells and pericytes provide an important cellular link between fibrosis and anemia development in CKD.[36]

Renal *EPO* mRNA expression and plasma EPO levels correlate directly with the number of EPO-producing cells (EPC) in the kidney.[37] Renal EPC are exquisite oxygen sensors that respond to small changes in tissue pO_2 with HIF-2α stabilization and increased *EPO* transcription.[25,38] In contrast to Hep3B hepatoma cells, which were used to purify HIF-1, the hypoxic induction of EPO in the kidney is HIF-2-dependent. This paradigm is based on genetic studies in mice, the analysis of human mutations, and on immunohistochemical examinations of rodent and human tissues.[39]

Our laboratory has recently shown that renal EPC are derived from forkhead box D1-expressing stroma, which surrounds the cap mesenchyme during kidney development and gives rise to essentially all nonendothelial renal interstitial cells including pericytes and perivascular fibroblasts, mesangial cells, and vascular smooth muscle cells. Interestingly, the ability to produce EPO is shared between brain and renal pericytes, which are both of neural crest origin.[38,40] PHD2 is the main prolyl hydroxylase that controls HIF activity under normoxic conditions. Its genetic inactivation in renal interstitial cells results in HIF-2 activation and EPO production in ~40% of cortical and outer medullary renal interstitial cells leading to severe polycythemia.[38]

HIF-2 also controls EPO synthesis in hepatocytes, which are the main source of EPO production during embryonic development. Although the liver does not contribute to plasma EPO levels in adults under baseline conditions, EPO synthesis in the adult liver can be reactivated by moderate to severe hypoxia, VHL, or PHD inhibition.[27,41,42] Furthermore, in the setting of end stage renal disease (ESRD) a significant fraction of plasma EPO is liver-derived.[43]

HIF coordinates erythropoiesis with iron metabolism

Stimulation of erythropoietic activity as a result of HIF-2-induced EPO synthesis increases iron demand in the bone marrow. To meet these requirements, iron uptake and utilization has to be adjusted (Figure 2). HIF-2 increases the transcription of *divalent metal transporter 1* (*DMT1*) and *duodenal cytochrome b (DCYTB)* and thus promotes iron uptake as demonstrated in animal models of iron-deficiency and hemochromatosis.[26,44,45] DMT1 transports iron into the cytoplasm of cells, and DCYTB reduces ferric iron to its ferrous form (Fe^{2+}) before it is taken up from the gut lumen into intestinal cells via DMT1 (Figure 2). In addition, HIF regulates the transcription of transferrin, which transports iron in its ferric form (Fe^{3+}) in blood, *transferrin receptor TFR1*,[46–48] *ceruloplasmin*, which oxidizes Fe^{2+} to Fe^{3+} and is also important for iron transport,[49] *heme-oxygenase-1*, which is involved in the recycling of iron from phagocytosed erythrocytes,[50] and *ferroportin* (*FPN*),[51] the only known cellular iron exporter.

FPN is targeted for degradation by hepcidin, a small 25-amino-acid peptide, produced by hepatocytes. Its production

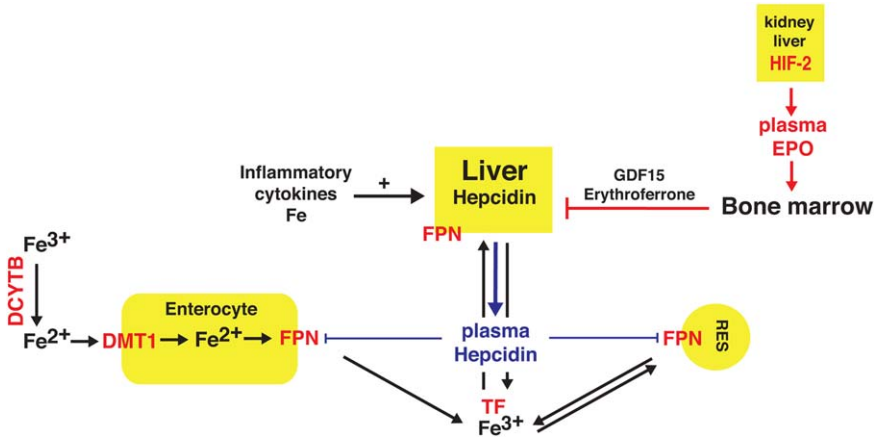

Figure 2 HIF-dependent regulation of iron metabolism. Schematic overview of HIF-regulated genes involved in iron metabolism shown in red. In the gut, duodenal cytochrome b (DCYTB) reduces ferric iron (Fe^{3+}) to Fe^{2+}, which enters enterocytes via the divalent metal transporter-1 (DMT1). *DCYTB* and *DMT1* are both bona fide HIF-2-regulated genes. Release of iron into the circulation occurs via ferroportin (FPN), which is hepcidin-regulated but also HIF-inducible. In blood, iron is transported in complex with transferrin (TF) to the liver, cells of the reticulo-endothelial system (RES), bone marrow and other organs. Increased erythropoietic activity in the bone marrow produces growth differentiation factor 15 (GDF15) and erythroferrone, which have been shown suppress hepcidin in hepatocytes. Inflammatory cytokines stimulate hepcidin production in the liver and lead to reduced ferroportin surface expression and hypoferremia.

is increased by iron and inflammatory cytokines such as interleukin 6. Because hepcidin suppresses FPN cell surface expression, high plasma hepcidin levels are associated with reduced intestinal iron uptake, and impaired release of iron from internal stores.[52] In patients with advanced CKD plasma hepcidin levels are frequently elevated and contribute to the pathogenesis of renal anemia as increased hepcidin leads to functional iron-deficiency.[36,53]

Systemic HIF activation suppresses hepcidin production in the liver enhancing iron uptake and mobilization.[54,55] Although initial studies suggested a direct role for HIF-1 in the suppression of hepcidin transcription,[56] subsequent analysis of mouse models with global or liver-specific HIF activation supported the notion that hepcidin suppression was dependent on EPO-mediated stimulation of erythropoiesis, i.e., that HIF does not act as a direct repressor of *hepcidin* transcription in the liver.[57,58] This notion is supported by genome-wide studies in cell lines.[19]

The relationship between HIF and iron metabolism is bidirectional, as iron itself can modulate HIF activation. The gene encoding HIF-2α contains an iron response element in its 5′ UTR, which binds iron regulatory proteins (IRPs).[59] Similar to the regulation of ferritin translation, the binding of IRPs to the *HIF2A* 5′ UTR has been shown to inhibit HIF-2α translation and thus decreases HIF-2α protein levels in the presence of low intracellular iron.[60–62] According to this model, renal EPO production would be relatively reduced in iron-deficiency anemia compared to other types of anemia with normal or elevated plasma iron levels.

In addition to regulating iron metabolism, hypoxia and the PHD/HIF pathway stimulate erythropoiesis through direct effects on the bone marrow via stimulation of EPO receptor expression, hemoglobin synthesis,[63–67] and modulation of stem cell maintenance, lineage differentiation, and maturation.[68,69]

HIF-PROLYL HYDROXYLASES AS DRUG TARGETS IN RENAL ANEMIA

Anemia associated with CKD is due to a relative deficiency in renal EPO production, concomitant functional, and/or absolute iron deficiency and resistance to EPO signaling, which is frequently seen in the setting of inflammation.[36] Pharmacologic activation of HIF signaling has the potential to provide a more comprehensive and physiologic approach to the treatment of renal anemia compared to therapy with recombinant EPO preparations alone. Recent clinical studies of compounds that target the PHD/HIF pathway are discussed below.

Rational for therapeutic targeting of the HIF system

The administration of erythropoiesis stimulating agents (ESAs), such as first-generation recombinant human EPO

(rhEPO) preparations epoetin alfa and epoetin beta, and second-generation epoetins darbepoetin alfa and epoetin beta pegol, is current standard of care for patients with renal anemia.[70–72] ESAs are effective in maintaining hemoglobin levels in target range in most patients and their use has reduced the need for red blood cell transfusions. Despite its clinical success, ESA therapy in advanced CKD is associated with an increased risk for cardiovascular events.[73–75] Although the underlying mechanisms are not clearly understood, ESA-associated cardiovascular events are most likely due to (a) intermittent supra-physiologic plasma EPO levels following the injections of high doses of ESAs to reach target hemoglobin levels, (b) hemoglobin oscillations, and (c) excursions of hemoglobin levels beyond target range.[76–80]

The identification of oxygen- and iron-dependent HIF-PHDs as key regulators of erythropoiesis has catalyzed the development of novel therapeutic agents, which are referred to as HIF-PHD inhibitors (HIF-PHIs). HIF-PHIs reversibly inhibit PHD catalysis and induce a transient increase in the expression of HIF-regulated genes, including renal, and hepatic *EPO*.[27] However, the increase of plasma EPO in CKD patients successfully treated with HIF-PHIs is significantly lower than in patients treated with injectable rhEPO.[81,82] Although clinical data are not yet available, HIF-PHI therapy holds the promise to reduce the cardiovascular risk associated with conventional ESA therapy. HIF-PHIs have also beneficial effects on iron homeostasis, as they lower hepcidin and ferritin and increase total iron binding capacity (TIBC) in patients with CKD.[81–86] Furthermore, pending additional investigation, HIF-PHIs have the potential to protect renal EPCs from loss of EPO production in chronic kidney injury, as suggested by preclinical studies.[87] Owing to their combined action on EPO synthesis and iron metabolism, and their ability to stimulate erythropoiesis without excessively raising plasma EPO levels, HIF-PHIs have the potential to become therapeutic alternatives to conventional ESA therapy.

HIF-PHIs in clinical trials

Several HIF-PHIs have been developed and are currently undergoing clinical investigation in nondialysis-dependent (NDD) CKD patients, EPO-naïve dialysis-dependent (DD) patients, and DD patients who were previously treated with ESAs (Table 1). Peer-reviewed clinical

Table 1 HIF-PHIs in clinical development for the treatment of anemia

Compound	Ref.	Development status	HIF-α stabilization	HIF-PHD targets
AKB-6548 (Vadadustat) Akebia Therapeutics	86,88,89	Phase 3	HIF-2α > HIF-1α	PHD3 > PHD2
GSK-1278863 (Daprodustat) GlaxoSmithKline	81,90–92	Phase 3	HIF-1α and HIF-2α	PHD2 and PHD3
FG-4592 (Roxadustat) Fibrogen/Astellas Pharma/ AstraZeneca	82,85	Phase 3	HIF-1α and HIF-2α	PHD1, 2 and 3
BAY 85-3934 (Molidustat) Bayer Pharma	93–95	Phase 2	HIF-1α and HIF-2α	PHD2 > PHD1/PHD3
JTZ-951 Japan Tabacco, Inc. /Akros Pharma, Inc.	96	Phase 2	Not published	Not published
Zyan1 Cadila Healthcare	97,98	Phase 1	Not published	Not published
JNJ-42905343 Janssen Pharmaceutica	99	Pre-clinical	HIF-1α and HIF-2α	PHD1, 2 and 3
DS-1093 Daiichi Sankyo, Inc.	–	Discontinued for anemia, under evaluation for other indications	Not published	Not published

The above table is based on a review of the literature and other publicly available resources such as patent applications. A direct comparison of half maximal inhibitory concentrations (IC50) of various compounds for HIF-PHDs, FIH, and other dioxygenases has not yet been published.

data from randomized controlled trials are available for three orally administered compounds, daprodustat (GSK-1278863), roxadustat (FG-4592), and vadadustat (AKB-6548) all of which have progressed to phase 3 clinical evaluations (Table 2). Others are currently in preclinical, phase 1 or 2 clinical trials for renal anemia or are being evaluated for other indications: Molidustat (BAY 85-3934, Bayer)[93]; JTZ-951 (Japan Tobacco, Inc./Akros Pharma, Inc.)[96]; JNJ-42905343 (Janssen)[99]; DS-1093 (Daiichi Sankyo, Inc., no longer investigated for anemia); Zyan1 (Cadila Healthcare Ltd.).[97,98] In addition to stimulating erythropoiesis, HIF-PHIs have been shown to produce nonerythropoietic effects, such as lowering cholesterol and reducing blood pressure.[81,82,84,93,100]

Daprodustat, Roxadustat, Vadadustat, and Molidustat are discussed in greater detail below. Vadadustat is the least potent inhibitor of PHD2, as the concentration needed to inhibit 50% of its activity in vitro (IC50) is the highest among the four compounds.

Daprodustat (GSK-1278863)

Daprodustat was developed by GlaxoSmithKline as an oral, once-daily HIF-stabilizing agent and is currently in phase 3 clinical trials for renal anemia. It contains a characteristic carbonylglycine side chain that is structurally analogous to 2OG. Preclinical studies demonstrated that daprodustat inhibits PHD2 and PHD3 leading to both HIF-1α and HIF-2α stabilization.[101] Holdstock et al. conducted a phase 2a study in 72 CKD patients not undergoing dialysis and not receiving rhEPO, and in 82 patients on hemodialysis previously treated with stable doses of rhEPO.[81] Participants in this 4-week study were randomized to either receive 0.5, 2, or 5 mg of once-daily daprodustat. Oral administration of daprodustat led to a dose-dependent increase in hemoglobin levels. In the NDD study group, a hemoglobin increase of 0.95 g/dL was observed with 5 mg daprodustat compared to a hemoglobin reduction of 0.23 g/dL in the placebo group. In the DD group, 5 mg of daprodustat was required to maintain hemoglobin levels in target range, whereas 0.5 and 2 mg were not effective. Median peak plasma EPO concentrations in the HIF-PHI-treated groups were much lower compared to DD patients receiving rhEPO (424.9.8 U/L vs. 24.7 U/L in the 5 mg DD group). Daprodustat treatment increased TIBC in both NDD and DD groups, whereas transferrin saturation (TSAT) was decreased in NDD patients. Hepcidin decreased in NDD patients in a dose-dependent manner, whereas no change (<0.5%) was observed in the DD group treated with 5 mg of daprodustat compared to the rhEPO control group. In addition to the effects on erythropoiesis and iron metabolism, Holdstock et al. reported dose-dependent decreases in total cholesterol, HDL-, and directly-measured LDL-cholesterol levels. Overall, the most common reported side effect was nausea in the NDD group and anemia in the DD arm (0.5 and 2.0 mg groups). Serious adverse events were reported in five patients of the NDD cohort (hypoglycemia, acute pancreatitis, acute renal failure, and azotemia) and four patients in the DD group (abnormal liver function tests, acute respiratory failure, constipation, and gastrointestinal hemorrhage). Deaths were not reported. Brigandi et al. reported a dose-dependent increase in plasma EPO and hemoglobin levels and a dose-dependent decrease in plasma hepcidin in a separate phase 2a study (daprodustat dose range from 10 to 100 mg).[90] The most common side effect in this study was also nausea, which was mainly observed in the high dose cohorts (50 and 100 mg).

Roxadustat (FG-4592)

Roxadustat is FibroGen's lead compound and has, in collaboration with Astellas Pharma and AstraZeneca, entered several large phase 3 cardiovascular outcomes trials. FG-4592 is structurally related to FG-2216, which is no longer used in humans due to a single case of fatal hepatic necrosis that was not attributed to the drug.[102] FG-2216 and FG-4592 are both hydroxy-quinoline-based compounds that differ structurally from each other by the addition of a phenoxy-substituent to the quinoline core of FG-2216.

Four major clinical studies have been published with Roxadustat. In the first study, Besarab et al. investigated the effects of roxadustat in 116 NDD-CKD patients treated orally BIW or TIW for 28 days at doses ranging from 0.7 to 2.0 mg/kg. This study demonstrated a dose-dependent increase in hemoglobin and decrease in plasma hepcidin, ferritin and TSAT were decreased at the end of study.[83]

Provenzano et al. published two dose-finding studies in NDD-CKD patients and in long-term hemodialysis patients on rhEPO therapy.[82,84] In the NDD study, 145 patients with hemoglobin ≤10.5 g/dL were enrolled and randomized into six different dosage regimen and treated with roxadustat for 16–24 weeks. 92% of roxadustat-treated patients responded with an increase in hemoglobin of ≥1 g/dL from baseline and hemoglobin level of ≥11.0 g/dL. This response was independent of baseline C-reactive protein levels and iron repletion status. Erythropoietic responses were associated with decreased hepcidin levels and iron supplementation was not needed to maintain hemoglobin. Furthermore, mean total cholesterol levels were reduced. Although 35 patients

Table 2 Summary of peer-reviewed clinical studies discussed in this review

Compound	Ref.	Pat	Durat weeks	Comp	Fe suppl	Hb	Ferritin	TIBC	TSAT	Hepcidin	Chol	VEGF
AKB-6548 Vadadustat	86	NDD	20	Placebo	Oral Fe allowed	+	↓	↑	No change	↓	No effect	No change
	88,89 (Meeting abstracts)	DD convers.	16	None, 3 dose cohorts	i.v. Fe permitted	+	n.r. $	n.r.$	n.r.$	n.r.$	n.r.$	n.r.$
GSK-1278863 Daprodustat	81	NDD	4	Placebo	Not specified	+	↓	↑*	↓*	↓	↓	No clear change#
		DD	4	rhEPO	Not specified	+ 5 mg only	↓ (5 mg)	↑*	No change (5 mg)	No change (5 mg)	↓	No clear change#
FG-4592 Roxadustat	83	NDD	4	Placebo	i.v. Fe prohibited, oral Fe allowed	+ Dose-dep.	↓ & Also placebo	↑	↓	↓	n.r.	n.r.
	85	DD Incident patients	12	None	One i.v., 2 oral and one no-iron cohort	+ Iron-indep.	↓	↑	↓	↓	n.r	n.r
	84	NDD	16–24	None, 6-cohort dose-finding study	i.v. Fe prohibited, oral Fe allowed	+ Starting dose-dep.	↓, for BL Ferritin ≥ 100 ng/mL	n.r.	↓ Initially	↓	↓	n.r.
	82	DD convers.	6 or 19	rhEPO	Oral Fe allowed, i.v. Fe as rescue	+	No change	↑ At 6 wks	No change	↓ At 19 wks	↓	n.r.

+ denotes effectiveness in changing or maintaining hemoglobin (Hb); *denotes trends, small, or statistically nonsignificant changes; # denotes that high interpatient variability was observed; &, not statistically significant compared to placebo; $, not reported in the meeting abstracts, manuscript containing iron data in preparation; BL, base line; convers., conversion from ESA therapy to HIF-PHI; Comp, Comparator; n.r., not reported; Pat, either dialysis-dependent (DD) or nondialysis-dependent (NDD) CKD patients, incident patients are newly initiated EPO-naïve hemodialysis or peritoneal dialysis patients.

experienced serious adverse events in this study, including 5 deaths (2 cardiopulmonary arrests, one pulmonary embolism, one cerebral infarction with pulmonary edema, and one unwitnessed death), none of these events were considered to be drug-related.[84] The second study was an active comparator study that consisted of two parts. In part 1, 54 hemodialysis patients were treated with roxadustat thrice-weekly for 6 weeks and compared to control patients treated with epoetin alfa. Part 2 consisted of a 19-week study of 90 patients randomized to 6 dosing groups and active comparator cohort. Intravenous iron was prohibited. Part 1 of the study showed a dose-dependent hemoglobin response and decrease in plasma hepcidin. In part 2 of the study, the average roxadustat dose requirement for maintaining hemoglobin $\geq$11.0 g/dL was $\sim$1.7 mg/kg and hepcidin levels decreased by $\sim$18%. Mean peak plasma EPO levels in patients receiving a mean roxadustat dose of 1.3 mg/kg were $\sim$130 U/L compared to $\sim$700 U/L in study participants receiving a median dose of 90 U/kg/wk of epoetin alfa.[82]

The fourth study by Besarab et al. investigated the efficacy of roxadustat treatment in EPO-naïve hemodialysis ($n = 48$) and peritoneal dialysis patients ($n = 12$).[85] Sixty patients were treated with roxadustat for 12 weeks (mean weekly dose range of 4.0–4.3 mg/kg administered TIW) in conjunction with oral iron, intravenous iron, or without iron therapy; 12 peritoneal dialysis patients received oral iron therapy. The study reported that the hemoglobin response in the iron-treated group was larger than in the noniron group, that hepcidin levels decreased between 40% and 80% and that no correlation was found between hemoglobin responses and C-reactive protein level prior to study begin.

Vadadustat (AKB-6548)

AKB-6548 is Akebia's lead compound. It is based on a hydroxy-pyridine core and contains a carbonylglycine side chain that is characteristic of other HIF-PHIs as well. AKB-6548/Vadadustat stabilizes HIF-2α to a greater extent than HIF-1α and produces a dose-dependent increase in plasma EPO levels.[103] It maintains hemoglobin levels in CKD patients, increases TIBC, and lowers plasma ferritin and hepcidin level without significant changes in baseline plasma EPO levels at the doses studied.[100,104] A slight decrease in mean arterial blood pressure was reported in a 28-day dose escalation study.[100] Akebia recently published the results of a 20-week, double-blind, randomized, placebo-controlled, phase 2b study in patients with stages 3a to 5 NDD-CKD.[86] The primary endpoint of this study was the percentage of patients who achieved or maintained a hemoglobin $\geq$11.0 g/dL or increased hemoglobin by $\geq$1.2 g/dL. The intent-to-treat study population consisted of 210 patients, 138 in the vadadustat, and 72 patients in the control group. Vadadustat was orally administered once-daily at a starting dose of 450 mg to ESA-naïve patients with hemoglobin $\leq$10.5 g/dL, patients who previously received ESA with hemoglobin $\leq$10.5 g/dL, or patients actively treated with ESA and hemoglobin $\geq$10.5 to $\leq$12.0 g/dL. Vadadustat was titrated to either 600 or 150 mg as per dosing algorithm and suspended for hemoglobin $\geq$13.0 g/dL. Vadadustat raised and maintained hemoglobin (primary endpoint) in 55% of patients compared to 10% in the placebo group. Hemoglobin excursions $\geq$13.0 g/dL occurred in 4% of patients. With regard to iron parameters, vadadustat decreased hepcidin, and ferritin levels and significantly increased TIBC. TSAT and serum iron were comparable between the vadadustat and the placebo group. VEGF levels were not different between the two groups, changes in total serum cholesterol, LDL-, and HDL-cholesterol or triglycerides were not reported. The overall incidence of adverse events was comparable between groups. Three deaths occurred in the vadadustat arm of the study; two deaths were considered as unrelated to the study medication and one death could not be further evaluated as it occurred at home and no autopsy was performed. Vadadustat was also evaluated in hemodialysis patients previously receiving ESAs (3 cohorts, starting doses of 300 mg QD, 450 mg QD, and 450 mg TIW). This 16-week open-label phase 2 study demonstrated that vadadustat maintained stabile hemoglobin levels in ESRD patients converted from injectable ESAs.[88] A correlation between baseline C-reactive protein levels at study entry and response to treatment with vadadustat was not observed. No serious adverse events were assessed as being related to vadadustat and no deaths were reported.[89] Because of these successful phase 2 trials, Akebia Therapeutics has recently launched two major phase 3 programs, PRO$_2$TECT in NDD-CKD patients, and INNO$_2$VATE to investigate vadadustat in DD-CKD patients

Molidustat (BAY 85-3934)

Bayer Healthcare compound 85-3934 (Molidustat) is structurally different from other HIF-PHIs in that it does not contain a carbonylglycine side chain. The compound is based on a dihydropyrazolone ring structure and has a moderate preference for PDH2. Two 16-week phase 2 studies have been completed, but are not yet published in peer-reviewed journals. Meeting abstracts indicate that molidustat effectively stimulated erythropoiesis in EPO-naïve NDD-CKD patients (fixed doses of 25, 50, or

75 mg once-daily, 25 or 50 mg BID) and NDD-CKD patients previously treated with ESAs (starting doses of 25, 50, and 75 mg with planned dose range between 15 and 150 mg; darbepoetin as active comparator).[94,95] The data on iron metabolism are not yet reported for molidustat. In addition to its effect on erythropoiesis, molidustat displayed antihypertensive and cardioprotective effects in rats that underwent subtotal nephrectomy.[93] Adverse events were relatively equally distributed between molidustat and control cohorts.

Potential adverse effects associated with HIF-PHI therapy

General considerations

The types of potential adverse effects associated with HIF-PHI therapy are frequently inferred from the analysis of genetically modified mice, which are characterized by the tissue-specific blockade or activation of HIF signaling. Although animal studies have provided important insights into cell- and context-specific HIF functions, the clinical consequences of systemic HIF activation in patients with CKD may be difficult to predict from these data. Genetic models with permanent HIF activation, e.g., as a result of VHL and PHD function loss, are often characterized by dramatic gene expression and functional changes, limiting their predictive value for clinical studies with short-acting HIF-PHIs that stabilize HIF-α transiently. Long-term preclinical studies in animals treated with HIF-PHIs would be more informative in this regard. In fact, one study assessed the effect of HIF-PHI roxadustat on tumor initiation, progression and metastasis in a VEGF-sensitive model of spontaneous breast cancer and did not report adverse outcomes.[105] Important information regarding the type and likelihood of adverse effects can also be gained from the study of patients with genetic mutations in the PHD/HIF pathway. For example, patients with Chuvash polycythemia are homozygous for a specific *VHL* mutation (R200W) that predisposes to the development of polycythemia and vascular complications such as cerebral vascular events and peripheral thromboembolism, but not the classic VHL-associated tumors, such as CNS hemangioblastomas, renal cysts, and clear cell carcinomas or pheochromocytomas.[106–108]

Adverse effects due to HIF activation

Although HIF-PHIs have been well tolerated in short-term clinical trials, there are several safety concerns relating to potential nonerythropoietic effects of repeated HIF activation on tissue homeostasis as HIF-1 and HIF-2 regulate a broad spectrum of cellular functions.[109] The extent of nonerythropoietic HIF effects produced by HIF-PHI administration are likely to be determined by the degree and duration of HIF activation, cell types involved, and the pharmacokinetic characteristics of HIF-PHIs.

Prolonged activation of the HIF system has profound effects on metabolic and cardiovascular homeostasis.[109] For example, HIF-2 has been implicated in the pathogenesis of pulmonary hypertension,[109–114] which raises concerns whether HIF-PHI therapy may adversely affect pulmonary artery pressure regulation in the CKD/ESRD patient population, which is characterized by a higher prevalence of pulmonary hypertension.[115] With regard to systemic blood pressure regulation, HIF-PHI therapy seems to be associated with a trend toward decreasing rather than increasing blood pressure.[93,100]

HIF is also a potent stimulator of angiogenesis as vascular endothelial growth factor (VEGF) and other pro-angiogenic factors are bona fide transcriptional targets of HIF. Increases in tissue VEGF levels may have pro-oncogenic effects and could exacerbate pathologic conditions that are characterized by vascular proliferation, such as diabetic retinopathy. Notwithstanding these concerns, increased plasma VEGF concentrations were not found in several phase 2 clinical trials.[81,86] However, it is unclear whether plasma VEGF levels alone can be used to assess these potential pro-angiogenic risks. The absence of elevated plasma VEGF levels may be due to relatively low levels of cellular HIF activation, which are sufficient to increase erythropoietic effects but not sufficient to increased VEGF expression. Daprodustat for example produces increases in plasma VEGF concentrations at doses $\geq$50 mg (up to 5 mg was used in the clinical studies published by Holdstock et al.).[91,116]

HIF has been shown to regulate glucose, fatty acid, cholesterol, and mitochondrial metabolism.[109,117] Some of the metabolic consequences of HIF activation are also seen in patients treated with HIF-PHIs. A decrease in serum cholesterol levels was reported in NDD-CKD and hemodialysis patients for daprodustat and roxadustat.[81,82,84] Furthermore, a trend for increased glucose was noted for daprodustat at high doses (50 and 100 mg).[90] To what degree intermittent HIF activation affects glucose homeostasis and fatty acid metabolism in diabetics and nondiabetics is not known and will need to be determined in long-term clinical trials. Preclinical studies in animal models indicate that the PHD/HIF axis plays an important role in pancreatic β-cell function.[118] This notion is consistent with genome-wide mRNA expression analysis, which showed that the HIF-1β subunit was expressed at very

low levels in pancreatic islets isolated from patients with type II diabetes.[119]

Safety concerns also exist with regard to HIF's role in the regulation of tumor cell growth and metastasis, as HIF signaling in malignant cells has been associated with tumor initiation and progression.[120]

Due to the short duration of phase 2 trials, the effects of HIF-PHI therapy on CKD progression and matrix turnover are not yet known. The role of HIF in CKD progression is controversial and long-term clinical studies are needed to address this issue.[121,122] A recent study in animal models investigated the role of hypoxia and HIF-1 signaling in the development of vascular calcifications. The study suggested that HIF-PHIs might enhance phosphate-induced vascular smooth muscle cell calcification in CKD or ESRD patients with poorly controlled mineral bone metabolism.[123] A molecular link also exists between HIF and FGF23 signaling, which may negatively impact cardiovascular risk in HIF-PHI-treated CKD patients.[124,125] Another potential adverse effect that may be observed in CKD patients on HIF-PHI therapy is hepatic toxicity. FG2216, the sister compound of roxadustat was taken out of clinical trials due to one case of fatal hepatic necrosis, although the death of the patient was not attributed to the study medication. To date, HIF-PHIs have been well tolerated and significant liver toxicity has not been reported in published peer-reviewed studies.

Adverse effects not related to HIF activation

In addition to HIF-mediated adverse effects, HIF-PHIs have the potential to interfere with cell signaling pathways that involve other non-HIF proline hydroxylation targets, non-HIF dioxygenases, or nonspecific effects that are not related to HIF signaling or inhibition of 2OG-dioxygenases. However, meeting reports suggest that HIF-PHIs daprodustat, roxadustat, vadadustat, and molidustat are relatively specific for HIF-PHDs and have little effect on factor inhibiting HIF (FIH) and dioxygenases involved in epigenetic gene regulation. FIH is an asparaginyl hydroxylase that modulates co-factor recruitment to the HIF transcriptional complex.[126–128]

In summary, notwithstanding the very promising performance of HIF-PHI therapy in short-term phase 2 clinical trials, more comprehensive physiologic studies and large long-term clinical trials are needed to better understand the functional and metabolic consequences of systemic HIF-PHI administration before HIF-activating compounds can be safely given to patients with CKD or ESRD for extended periods of time.

CONCLUDING REMARKS

Here, I have provided a brief and concise overview of oxygen-dependent mechanisms that regulate erythropoiesis and iron metabolism in the context of renal anemia. The discovery of the PHD dioxygenases as key regulators of HIF signaling and erythropoiesis has led to the development of novel therapeutic agents that harness hypoxia responses for therapeutic gain. These HIF-activating compounds are currently in phase 3 clinical development for the treatment of renal anemia and have the potential to significantly impact the clinical practice of Nephrology.

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