Published in final edited form as:

Expert Opin Drug Discov. 2019 July ; 14(7): 667–682. doi:10.1080/17460441.2019.1613370.

The Transcriptional Factors HIF-1 and HIF-2 and Their Novel Inhibitors in Cancer Therapy

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Abstract

Introduction: Hypoxia is one of the intrinsic features of solid tumors and it is always associated with aggressive phenotypes, including resistance to radiation and chemotherapy, metastasis, and poor patient prognosis. Hypoxia manifests these unfavorable effects through activation of a family of transcription factors, Hypoxia- inducible factors (HIFs) play a pivotal role in the adaptation of tumor cells to hypoxic and nutrient-deprived conditions by upregulating the transcription of several pro-oncogenic genes. Several advanced human cancers share HIFs activation as a final common pathway.

Areas covered: This review highlights the role and regulation of the HIF-1/2 in cancers and alludes on the biological complexity and redundancy of HIF-1/2 regulation. Moreover, this review summarizes recent insights into the therapeutic approaches targeting the HIF-1/2 pathway.

Expert opinion: More studies are needed to unravel the extensive complexity of HIFs regulation and to develop more precise anticancer treatments. Inclusion of HIF-1/2 inhibitors to the current chemotherapy regimens has been proven advantageous in numerous reported preclinical studies. The combination therapy ideally should be personalized based on the type of mutations involved in the specific cancers and it might be better to include two drugs that inhibit HIF-1/2 activity by synergistic molecular mechanisms.

Keywords

Hypoxia; Hypoxia-inducible factors; HIF-1α; HIF-2α; HIF-3α; Hypoxia response elements; HIF-1 inhibitors; HIF-2 inhibitors; chemoresistance; radioresistance; angiogenesis

1. Introduction:

Hypoxia and necrosis are two characteristic features of solid tumors. Tumor cells develop hypoxia as a result of inadequate supply of oxygen (chronic hypoxia) or transient fluctuation in blood flow (acute hypoxia)[1]. The impairment in diffusion, the abnormalities in the tumor microvessels and the disturbed microcirculation, all lead to deficiency or even abolishment

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Declaration of Interest:

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in oxygen supply in the tumor microenvironment[2]. Eventually, tumor cells become necrotic due to lack of oxygen. Hypoxia negatively influences the results of radiotherapy and chemotherapy and potentiates tumor metastasis. This is well supported by scientific evidence from early 1950s as in 1953, Gray and co-workers showed that the therapeutic response of tumor cells to radiation in a well-oxygenated medium is better than when the tumor cells are irradiated under anoxic conditions[3]. However, these findings could be explained based on the crucial role of oxygen in the success of radiation therapy. Oxygen interacts with the free electron in the DNA that formed because of the radiation therapy. This oxygen-free radicals interaction makes the radiation damage permanent, and this explains the radioresistance of hypoxic cells which have low level of oxygen[4]. The impact of hypoxia on chemoresistance can be attributed to several factors. First, low drug concentration in hypoxic cells as it accumulates in areas away from functioning blood vessels[4]. Second, most anticancer drugs target proliferating cells; however, hypoxic cells experience nutrient starvation and impaired cell proliferation compared to aerobic cells, and thus they have less effect on hypoxic cells[4, 5]. The unfavorable effects of hypoxia extend beyond its negative impact on the effectiveness of radiotherapy and chemotherapy, as hypoxic microenvironment is linked with genomic instability, genetic alterations, mutagenesis, and poor prognosis[6]. For instance, hypoxia is able to select for cells expressing mutations in both p53, a tumor suppressor gene, and Bcl-2, an apoptosis-inhibiting protein, in oncogenically transformed cells[7]. By doing so, hypoxia contributes to solid tumor malignancy and metastasis. Hypoxia either locally or systemically provokes acute (short-term) and long-term responses in a number of physiologically relevant genes and mediates the switch from aerobic to anaerobic energy metabolism. Hypoxia induces the transcription of over 40 genes involved in various cellular functions including vascular architecture and tone: vascular endothelial growth factor (VEGF), red blood cell production (erythropoietin, EPO), iron metabolism (transferrin), energy metabolism that mainly includes the glucose transport proteins (GLUT-1, GLUT-3), insulin like growth factor-2 (IGF-2), and pH regulation (carbonic anhydrase-9, CA IX)[8, 9]. The overall goal of these various activation mechanisms is to increase oxygen delivery and decrease oxygen consumption or activate alternative metabolic pathways that do not require oxygen[10, 11]. However, these oxygen-regulated genes bear a specific protein sequence known as hypoxia-responsive elements (HREs) that specifically bind to Hypoxia-inducible factors-1–3 (HIF-1–3). HIFs are intrinsic markers of tumor hypoxia in which their expression are increased in hypoxic cells as a means of an adaptive response to hypoxic environment and tumor progression and metastasis.

2. The three HIF isoforms: their structures and functions:

2.1. HIF-1α:

HIFs is a family of three members and they are heterodimers composed of an O_2 sensitive α subunits (HIF-1α, or HIF-2α or HIF-3α) and an O_2 insensitive HIF-1β subunit[12]. HIF-1α is the most well-characterized isoform of the HIFs, and most of the current knowledge on the structure and the regulation of these transcriptional factors are based on the studies of mammalian HIF-1α and to a lesser extent, HIF-2α. HIF-1α, a 120-kDa polypeptide subunit, heterodimerizes with HIF-1β which is a 91- to 94-kDa polypeptide subunit, to form the transcription factor HIF-1. Both are classified as a member of the basic helix-loop-helix/Per-

Arnt-Sim (bHLH/PAS) family of transcription factors because both HIF-1α and HIF-1β exhibit regions of amino acid sequence homology with Per (a Drosophila circadian rhythm protein) and Sim (a protein involved in Drosophila central nervous system development). However, they contain one bHLH domain and two PAS domains (PAS-A and PAS-B). The HLH and PAS domains mediate heterodimerization between HIF-1α and HIF-1β, while the basic regions, prior to the N terminus of the HLH domain are responsible for the binding of the HIF-1α/HIF-1β heterodimer to the HREs-DNA motif of target gene promoters (Figure 1)[13, 14]. In addition to these domains, HIF-1α has two transactivation domains (TAD), which are functionally distinct from the DNA-binding and heterodimerization domains. These TAD are located at the C-terminal half of the HIF-1α, not within their N-terminal portion, which contains their bHLH and PAS domains[13]. Both hypoxia responsiveness and transactivation capability of HIF-1α reside within its C-terminal region[14, 15]. These independent transactivation domains are: the NH_2-terminal transactivation domain (N-TAD) localized to amino acids residues (531–575) in humans and the COOH terminal transactivation domain (C-TAD) localized to amino acids residues (786–826) in humans. Consistent with other previously described transactivation domains, both N-TAD and C-TAD are rich in acidic and hydrophobic residues[16]. The two transactivation domains are separated by (576–785) amino acid sequences in humans known as the inhibitory domain (ID) and this negative regulatory domain suppresses the transcriptional activity of N-TAD and C-TAD in normoxic conditions[15, 16]. The hypoxia-inducible transactivation capability of HIF-1 is mainly dependent on its HIF-1α subunit since HIF-1α is the oxygen labile subunit and contains the transactivation domains which are responsible for HIF-1α transcriptional activity. Whereas, HIF-1β is dispensable for the induction and serves as a dimerization partner[1417]. The transcriptional activity of HIF-1α is regulated by the oxygen cellular tension[16, 18]. The C-TAD regulates the transactivation of target genes through co-activators recruitment, co-activators CBP and p300[19]. The N-TAD is located within a region known as the oxygen-dependent degradation domain (ODDD) which is localized to the human HIF-1α amino acid residues (400–600). The ODDD is recognized by von Hipple-Lindau (pVHL) only under normoxic conditions and pVHL is required for mediating the HIF-α degradation by ubiquitin-proteasome pathway[15, 20]. The ODDD contains two key proline residues targeted for hydroxylation in normoxic conditions, thus; the ODDD controls the activity and stability of the α subunit and the carboxyl terminal region of HIF-1α represents the protein stability domain[15, 21, 22]. The ODDD and N-TAD regions are present only in HIF-1α which is unlike HIF-1β where HIF-1β contains C-TAD only. Both HIF-1α and HIF-1β mRNAs are constitutively expressed in various human tissues, however; HIF-1α mRNA levels show steady-state expression regardless of cellular oxygen tension and are not induced by hypoxia in most tissue culture cell lines[13, 23, 24]. In contrast to what have been observed *in vitro*, HIF-1α mRNA levels increase significantly in response to hypoxia in brain, heart, kidney, lungs, and skeletal muscle *in vivo*[25]. Nonetheless, HIF-1α and HIF-1β are also differ in that HIF-1β protein is constitutively active and stable in both aerobic and hypoxic cells whereas HIF-1α protein is degraded rapidly under normoxic conditions by the ubiquitin-proteasome system[11, 26]. Thus, the stability of HIF-1α is the primary determinant for the regulation of HIF-1 activity[23]. Hence, HIF-1α is a conditionally regulated transcription factor whereas HIF-1β is a constitutively active transcription factor. HIF-1β is also different from HIF-1α in its ability to heterodimerize with other proteins such as Aryl hydrocarbon receptor (AhR)

and SIM and this is owed to the fact that HIF-1β is identical to the Aryl hydrocarbon receptor nuclear translocator (ARNT) protein, which is required for the dioxin-AhR function[13]. In addition, HIF-1β can homodimerize *in vitro* unlike HIF-1α.

2.2. HIF-2α:

HIF-2α and HIF-3α are two closely related homologues of HIF-1α (Figure 1). HIF-2α was reported by groups of researchers around the same time and it was previously denoted by different names: Endothelial PAS domain protein 1 (EPAS1), HIF-1α-like factor (HLF), HIF-1α related factor (HRF) and member of the PAS superfamily-1 (MOP-1)[27–30]. HIF-2α shows 48% amino acid sequence homology overall with HIF-1α and it has a similar domain arrangement[21, 27, 28]. Although HIF-1α and HIF-2α share very similar characteristics including their abilities to heterodimerize with HIF-1β, binding to hypoxia inducible genes bearing HREs motif, and transcriptional activation, they are different in their expression levels in different tissues during different developmental stages[21, 27–30]. HIF-2α is expressed most abundantly in embryonic development stage and adult vascular endothelial cells, lungs, placenta and heart, whereas; HIF-1α has a ubiquitous expression in all analyzed mammalian tissues and cell types, specifically heart and kidney[25, 28, 30, 31]. HIF-1α and HIF-2α show different specificity in their transcriptional targets. For instance, HIF-1α effectively stimulates the expression of glycolytic enzymes, such as Lactate dehydrogenase-A (LDH-A) and CA IX. In contrast, HIF-2α acts more effectively on EPO gene and genes involved in iron metabolism while another group of genes, including VEGF and GLUT-1, are regulated by both HIF-1α and HIF-2α[32, 33].

2.3. HIF-3α:

HIF-3α (long HIF-3α variant) was firstly reported as a new bHLH-PAS protein in mice with 662 amino acids and a molecular weight of 73 kDa[34]. In the same paper, Gu and co-authors showed that HIF-3α has 57% and 53% amino acid sequences identity in the bHLH-PAS domain with HIF-1α and HIF-2α respectively, and 61% identity in the ODDD with HIF-1α. The first human HIF-3α (667 amino acid sequence) (Figure 1) was reported in 2001 with a high similarity with human HIF-1α and HIF-2α in the bHLH and PAS domains, and it contains N-TAD but lacks C-TAD transactivation domain. Interestingly, another HIF-3α was showed to contain a leucine zipper (LZIP) domain in the place of the C-TAD, which mediates DNA binding and protein-protein interaction[35, 36]. The expression pattern of HIF-3α is distinct from that of HIF-1α and HIF-2α. HIF-3α is expressed in adult mice thymus, lung, brain, heart and kidney. Similar to HIF-1α and HIF-2α, HIF-3α is shown to heterodimerize with HIF-1β *in vitro* and *in vivo*[34]. A lot of evidences suggest that the expression and the activity of the mouse, rat and human HIF-3α are enhanced in response to hypoxia[37, 38]. Moreover, other evidence suggests that HIF-2α plays a role in upregulating the expression and the transcriptional activity of the HIF-3α mRNA[39]. Another spliced variant of HIF-3α (short HIF-3α variant) in mice was reported as a negative regulator of HIF mediated expression of hypoxia inducible genes and it was designated as Inhibitory PAS domain protein (IPAS)[40]. Nevertheless, IPAS is N-TAD and C-TAD deficient and it is unique in its ability to act as repressor of hypoxia induced transactivation function of HIF-1α and HIF-2α, which adds to the complexity in the regulation of hypoxia-inducible genes by the HIFs. IPAS exerts its inhibitory action through physical interaction with the

bHLH/PAS domain of HIF-1α and this interaction results in the formation of dysfunctional complex with HIF-1α in the nucleus[41]. In contrast to HIF-1α, IPAS shows a restricted pattern of tissue expression with rigorous expression in Purkinje cells of the cerebellum and corneal epithelium of the mice. This extensive expression of IPAS in the cornea has its biological significance where IPAS counteracts the function of HIF-1α in the cornea and suppresses the corneal epithelium VEGF gene activation and neovascularization, and this is important in maintaining corneal transparency which is required for clear visions. Interestingly, IPAS is preferentially singled out HIFs without affecting other members of the bHLH/PAS transcription factor family such as AhR[40]. The selective negatively regulation of IPAS on HIF-1α could be exploited in compromising tumor angiogenesis and other pathological conditions associated with elevated activation of HIF-1α. Yuichi Makino *et al.* have shown that IPAS-expressing hepatoma cells produced tumors with slower growth rate and lower vascular density relative to the wild type cells[40]. However, it was shown that HIF-1α binds to the HREs in the promoter region of the IPAS gene and mediates hypoxia-dependent activation of gene transcription[42]. Likewise, Neonatal and embryonic PAS (NEPAS) is another splice variant of mouse HIF-3α, which is expressed predominantly during late embryonic and early postnatal stages. It acts as a negative regulator of HIF-1α and HIF-2α mediated gene expression where it can dimerize with HIF-1β and indirectly inhibit HIF-1α and HIF-2α activity[43]. Other multiple splice variants of the human HIF-3α have also been reported[36, 44, 45], however; it was evident that all human HIF-3α variants are induced by hypoxia and the induction is mediated by HIF-1α but not by HIF-2α[45]. In contrast, it worth mentioning that contradicting evidence was published concerning the expression of both the mRNA and protein levels of adult and embryos zebrafish *hif-3α* where its expression was increased by hypoxia and it was not directly regulated by HIF-1α[46]. Moreover, the hypoxic regulation of HIF-3α mRNA levels is tissue-specific in zebrafish unlike in the mammals[46].

4. HIF-1/2α regulation pathways:

4.1. Canonical mechanisms regulating HIF-1/2α:

4.1.1: Hydroxylation: It is well established that transcriptional activity and stability of HIF-1/2α are tightly regulated by oxygen-dependent hydroxylation of their α subunits, where normoxia leads to quick degradation of HIF-1/2α transcript. Conversely, Hypoxia stabilizes HIF-1/2α via inactivation of pVHL, thus decreases HIF-α ubiquitination and proteasomal degradation (Figure 2)[47, 48]. pVHL mediates the assembly of a complex composed of VHL, Elongin B, Elongin C and a catalytic RING subunit (RBX1), which binds ubiquitin-conjugated E2 component, and it is organized on a cullin scaffold protein (CUL2) to accomplish ubiquitination of VHL-bound HIF-1/2α proteins. However, this ubiquitination step requires a posttranslational hydroxylation step of two separate consensus proline residues (P402 and P564) within the ODDD of the human HIF-1α and (P405 and P531) with in the ODDD of human HIF-2α subunits[49, 50]. Here, prolyl hydroxylases domain enzymes (PHD1–4), most prominently PHD2, represent an essential component of the canonical regulation pathways of HIF-1/2. They utilize molecular oxygen, α-ketoglutarate, ascorbate, and iron as substrates and generate CO_2 and succinate as by-products. Beside the prolyl hydroxylases, Factor inhibiting HIF (FIH), an asparaginyl hydroxylase, regulates

HIF-1/2α transcriptional activity under normoxic conditions by hydroxylating the asparagine 803 and asparagine 847 residues within the C-TADs of HIF-1α and HIF-2α respectively and blocking the interaction between HIF-1/2α and transcriptional co-activators CBP/p300[19, 51]. FIH is also an iron and α-ketoglutarate dependent dioxygenase and is activated only in the presence of molecular oxygen. Therefore, hypoxia inhabits the functions of PHDs and FIH, stabilizes HIF-1α and HIF-2α and their transcriptional activity. Parallel to limited oxygen availability caused by hypoxia, inhibition of PHDs and FIH, and inactivation of pVHL are also owed to the oxidation of PHDs and FIH, which contain Fe (II) in their active sites, by reactive oxygen species (ROS) generated in the mitochondria through electron transport Complex III[52]. However, other ROS generating mechanisms that might stabilize HIF-1/2α have been reported such as NADPH oxidase systems and specifically the Nox family of NADPH oxidases. NADPH oxidase 1 (also known as NOX1) mediates ROS production as a mechanism to upregulate HIF-1α[53]. Whereas, NOX1 and NADPH oxidase 4 (also known as NOX4) help maintain HIF-2α expression in renal carcinoma via ROS generation and therefore, contribute to renal carcinogenesis[54].

4.2. Non-canonical mechanisms regulating HIF-1/2:

Although the oxygen dependent pVHL pathway provides a major regulatory mechanism for HIF-1/2α protein stability, additional mechanisms do exist to fine-tune the HIF-1/2α protein stability, synthesis, and transcriptional activity in both hypoxia and normoxia. However, because our understanding for the biology of the oxygen-independent control of HIF-1α is becoming clearer than its isoform HIF-2α, the oxygen-independent control of HIF-2α is needed to be investigated in more details.

4.2.1: Acetylation and deacetylation: Many posttranslational acetylation and deacetylation events have been reported to play a role in regulating both HIF-1/2α protein stability and transcriptional activity. However, conflicting data bring into question about the foundations of these regulation mechanisms and their roles in HIF-1/2α regulation require clarification. Multiple sites of the HIF-1α protein can be modified by lysine acetylation leading to different downstream effects. For example, acetylation within the ODDD is related to the pVHL-dependent HIF-1α degradation where Jeong *et al.* showed that pVHL binding is also promoted by acetylation of lysine (K532) residue of HIF-1α by direct binding of Arrest defective-1(ARD1), a protein acetyltransferase[55]. Thereafter, the ubiquitinated HIF-1α serves as the signal for degradation mediated by the 26S proteasome. However, the acetylation function of ARD1 is counteracted by the action of Metastasis-associated protein 1 (MTA1), where MTA1 induces the deacetylation of HIF-1α at K532R by increasing the expression of Histone deacetylase 1 (HDAC1) and thus enhances the transcriptional activity and stability of HIF-1α protein. In addition, the expression of MTA1 is strongly induced under hypoxic conditions and it is physically associated with HIF-1α when they are co-expressed[56]. Therefore, both MTA1 and HIF-1α are expected to have important roles in tumor metastasis and progression. Similarly, Zhu *et al.* showed that Metastasis-associated protein 2 (MTA2), another member of the MTA family, deacetylates HIF-1α and enhances its stability through interacting with HDAC1 in pancreatic carcinoma[57]. Yet, Arnesen *et al.* and Murray-Rust *et al.* reported that K532R mutation did not affect the interaction between the HIF-1α ODDD and human ARD1 (hARD1), and they

concluded that hARD1 did not acetylate and destabilize HIF-1α[58]. Moreover, Fisher *et al.* showed that inhibition and overexpression of ARD1 did not affect basal HIF-1α levels or its response to hypoxia[59]. Whereas, acetylations of lysine (K709) and lysine (K674) in the carboxy terminal region of HIF-1α are related to HIF-1α/p300 interaction and HIF-1 transactivation. For example, Geng *et al.* demonstrated that p300, a component of the HIF-1 transcriptional complex, stabilizes HIF-1α via acetylating lysine (K709) residue in both normal and hypoxic conditions, and they showed that this acetylation is opposed by HDAC1[60]. However, K674 in HIF-1α was shown to be acetylated primarily by the CBP/p300 -associated factor (PCAF) leading to the increase of HIF-1α protein levels and binding of p300[61]. Moreover, in the same study, Lim *et al.* showed that Sirtuin 1 (SIRT1), a NAD-dependent deacetylase, binds to HIF-1α, deacetylates it at K674 position, blocks p300 recruitment and consequently represses HIF-1 target genes. Conversely, Dioum *et al.* and Chen *et al.* demonstrated that HIF-2α can be acetylated at K385, K685, and K741 positions within its C terminus by CBP and selectively deacetylated by SIRT1 to augment HIF-2 signaling[62, 63].

4.2.2: PI3K/AKT and MAPK/ERK pathways: In non-hypoxic conditions, overexpression of HIF-1α could be achieved by growth factors stimulation where they are able to increase HIF-1α protein synthesis in a cell type-specific manner via activation of protein tyrosine kinases (PTKs) by mutation or ligand binding. This activation leads to signaling via the phosphatidylinositol 3- kinase (PI3K)/protein kinase B (AKT) pathway or mitogen-activated protein kinase (MAPK/ERK) pathway (Figure 3). The two pathways are affected by the tumor microenvironment favorable selection of cells with somatic mutations that activates oncogenes and inactivate tumor suppressor genes. These mutations drive cells through the cell cycle in an uncontrolled manner and prevent apoptosis. PI3K regulates HIF-1α protein synthesis via its target downstream serine threonine kinases, AKT and rapamycin-associated protein (FRAP/FKBP), which is also known as mammalian target of rapamycin (mTOR). FRAP/mTOR mediates its action via phosphorylation of two downstream effectors, the translational regulatory proteins eIF-4E binding protein 1 (4E-BP1) and p70 ribosomal protein S6 kinase (p70S6K). Phosphorylation of p70S6K phosphorylates its substrate, the ribosomal protein S6 (rpS6) and phosphorylation of 4E-BP1 disrupts its inhibitory interaction with eukaryotic translation initiation factor 4E (eIF-4E)[64] and eventually these phosphorylation actions result in enhanced translation of HIF-1α mRNA into protein. While HIF-1α expression is dependent on both mTOR complex 1 (mTORC1) and mTOR complex 2 (mTORC2) in renal carcinoma cells, HIF-2α expression is solely dependent on mTORC2[65]. The mTORC1 and mTORC2 represent two functionally distinct mTOR-containing signaling complexes, where mTORC1 phosphorylates p70S6K and 4E-BP1 and mTORC2 phosphorylates AKT. Similarly, the activation of the RAS-RAF-MEK-ERK pathway, where activated ERK1 and ERK2 phosphorylate and activate the MAP kinase-interacting kinases 1 and 2 (MNK1 and MNK2), which directly phosphorylate and stimulate the activity of eIF-4E and thus enhancing HIF-1 α translation. Accordingly, IGF-1[66], IGF-2, Epidermal growth factor (EGF), and Fibroblast growth factor 2 (FGF-2) as well as autocrine activation of the IGF-1 and IGF-2 receptors induce HIF-1α expression[66, 67] and protein synthesis via activation of both the PI3K/AKT and MAPK pathways[66, 68]. Likewise, IGF-1 was reported to induce the transcription of HIF-2α via

PI3K-mTORC2 system and promote vascularization in neuroblastoma[69]. Interestingly, HIF-1 is a transcriptional activator of IGF-2 which is the most highly upregulated gene in colon cancer[70]. However, increased activity of the HER-2 (also known as neu) tyrosine kinase receptor in breast cancer is associated with increased HIF-1α protein and VEGF mRNA expression via activation of a signal transduction pathway of PI3K/AKT[71, 72]. In prostate carcinoma and glioblastoma, mutation and inactivation of the tumor suppressor PTEN, which acts as a negative regulator of the PI3K via dephosphorylating the 3 position of phosphoinositides, is evident with elevated HIF-1α protein expressions[73–75]. In addition to PTEN, VHL and p53 are two tumor suppressor genes in which their loss of function mutations results in increased expression of HIF-1α. p53 is known to recruit the murine double minute 2 (Mdm2), an E3 ubiquitin–protein ligase to degrade HIF-1α[76]. Jun activating binding protein 1 (Jab1), also known as constitutive photomorphogenic-9 (COP9) signalosome subunit 5 (CSN5), counteracts the effect of p53 by competing with p53 to bind directly to the ODDD of HIF-1α. Jab1/CSN5 stabilizes HIF-1α by blocking hypoxia dependent p53-mediated degradation and promotes the transcriptional activity of HIF-1α[77]. However, activating mutations in oncogenes such as the previously mentioned MTA1 is associated with increased HIF-α protein level and VEGF protein level, progression and metastasis of the pancreatic cancer[78]. V-Src is another oncogene where its overexpression increases the expression of HIF-1α protein level without affecting its transactivation domain function, and thus increases the transcriptional activation of genes encoding VEGF and enolase 1 (ENO1)[79] via increased activity of both the PI3K/AKT pathway and HIF-1α[80].

4.2.3: HIF-1/2α and other phosphorylation events: In addition to regulating HIF-1α protein synthesis, the MAPK/ERK pathway is also implicated in HIF-1α transcriptional activation, where ERK is reported to phosphorylate the co-activator CBP/p300 and increase HIF-1α/p300 complex formation[81]. Moreover, ERK2 was reported to directly phosphorylate the C-terminal domain of HIF-1α at two distinct serine residues (S641 and S643) and by doing so it blocks HIF-1α nuclear exclusion by Chromosomal maintenance 1 (CRM1 also known as Exportin 1, XPO1) and enhances the nuclear accumulation and activity of HIF-1α[82]. However, Gradin *et al.* showed that HIF-1α and HIF-2α were phosphorylated at threonine 796 and threonine 844, respectively, under hypoxic conditions. This phosphorylation step increased the affinity of the interaction between HIF-1/2α and the transcriptional co-activator CBP/p300[83]. Moreover, Lancaster *et al.* showed that phosphorylation of HIF-1α at threonine 796 prevented the hydroxylation of asparagine 803 by FIH[84]. However, HIF-1α can be phosphorylated at several threonine and serine residues including: threonine 63 and serine 692 by Protein kinase A (PKA)[85], serine 696 by Ataxia telangiectasia mutated serine/threonine kinase (ATM)[86], and serine 668 by Cyclin-dependent kinase 1 (CDK1)[87]. Whereas these modifications by PKA, ATM and CDK1 resulted in increased HIF-1α stability, phosphorylation by Glycogen synthase kinase 3β (GSK3β) at serine 551, threonine 555, and serine 589[88], or by polo-like kinase 3 (PLK3) at serine 576 and serine 657 increases HIF-1α degradation[89]. HIF-2α is phosphorylated at by casein kinase 1delta (CK1δ) at serine 383 and threonine 528, and as a result, CK1δ enhances the nuclear accumulation of HIF-2α via blocking the CRM1-dependent export of HIF-2α from the nucleus[90]. On the contrary, CK1δ phosphorylates HIF-1α at serine 247 and inhibits its activity via inhibiting its ability to associate with HIF-1β[9192].

4.2.4: Hsp90, HAF and Hsp70: HIF-1α, like other bHLH-PAS protein such as AhR and Sim, is stably associated with the Heat shock protein 90 (Hsp90), a 90 kDa molecular chaperone that assists the covalent folding and assembly and controls stabilization of several client proteins. Hsp90 is involved in HIF-α subunit stabilization against non-pVHL mediated ubiquitination and proteasomal degradation and it aids HIF-1α accumulation in the nucleus. It binds to the PAS domain and stabilizes the HIF-α subunit predominantly under normoxic conditions, and it is displaced by HIF-β subunit primarily under hypoxia following nuclear translocation[93, 94]. Hsp90 competes with the Receptor of activated protein kinase C1 (RACK1) for binding to the HIF-1α PAS domain since the interaction between HIF-1α and RACK1 destabilizes HIF-1α via proteasomal degradation pathway in an oxygen-independent manner[95]. Moreover, Hsp90 appears to chaperone a proper conformation of the HIF-1 and facilitates its binding to HREs motif on the DNA of target genes[96]. Interestingly, whereas acute exposure to hypoxia resulted in increased HIF-1α accumulation, hypoxia could cause degradation and decrease of the accumulation of HIF-1α, but not HIF-2α protein levels in cell culture systems upon prolonged exposure. Mei Koh and colleagues have shown that selective oxygen-independent degradation of HIF-1α and promotion of HIF-2α transactivation are controlled by Hypoxia-associated factor (HAF; also known as SART1). HAF is an E3 ubiquitin ligase targets HIF-1α specifically for proteasomal degradation following prolonged hypoxia. HAF also binds to a different site on HIF-2α although it increases HIF-2α transactivation without causing its degradation. Thus, HAF represents a switching mediator for the hypoxic response of the cancer cell from HIF-1α-dependent to HIF-2α-dependent and provides an elucidation for enhanced tumor progression under prolonged hypoxia[97, 98]. However, Heat shock protein 70 (Hsp70) was reported to have a role in the prolonged hypoxia mediated HIF-1α degradation but not HIF-2α. Hsp70 recruits the ubiquitin ligase, carboxyl terminus of Hsp70-interacting protein (CHIP) to selectively promote the ubiquitination and proteasomal degradation of HIF-1α but not HIF-2α[99]. Consequently, the literatures suggest the opposite roles of Hsp70 and Hsp90 in regulating HIF-1α where Hsp70 and Hsp90 may be involved in degradation and stabilization of HIF-1α, respectively.

5. HIF-1/2 transcriptional co-activators:

Hypoxia inducible transcription of various hypoxia-responsive genes is not solely dependent on HIF-1/2 binding; however, optimal gene transcription requires HIF-1/2 to interact with adjacent, or sometimes distant, transcription factors and co-activating proteins to form multiprotein complexes[100, 101]. These multiple interacting transcription factor binding sites are different in each oxygen-regulated gene and each cell type, which convey some degree of tissue selectivity and contribute to the unique regulation of that gene with respect to its level of induction by hypoxia[100]. Moreover, they play a role in HIF-1/2 target gene specificity since many reported HIFs co-activators exhibit specific physical interaction with only one HIF isoform, and thus preferentially enhancing only one HIF isoform's activity in the correct gene promoter context. Interestingly, being an essential part in HIF-1α or HIF-2α transcriptional complexes, they may provide additional therapeutic targets for anticancer treatments. However, several co-activators have been previously reported to enhance HIF-1/2 ability to activate target genes expressions through stabilizing the interaction of HIF-1/2 with

the transcriptional co-activator CBP or p300. The activation of Endothelin-1 (ET-1) promoter expression by hypoxia in endothelial cells is one example where it has a three adjacent transcription factor binding sites: Activator Protein-1 (AP-1), GATA-binding factor 2 (GATA-2) and Nuclear Factor-1(NF-1), and they form a functional hypoxia responsive complex with HIF-1. AP-1, GATA-2, and NF-1 to stabilize the binding of HIF-1 and promote recruitment of CBP/p300 to the HIF-1 hypoxia response complex[102]. Hypoxia, along with anemia, induces the synthesis of the EPO in liver and kidney. The function of EPO enhancer in hypoxic liver tissue is shown to be modulated by sequences lying 3′ downstream of HREs which contains the HIF binding site. These 3′ sequences co-operate to permit the action of the inducible hypoxia at a distance from EPO enhancer and they have been identified as being necessary for hypoxic induction[101, 103]. Hepatocyte nuclear factor-4 (HNF4) was reported to bind to the region of EPO enhancer and was shown to physically interact with the HIF-1β subunit of the dimeric HIF-1 and cooperate with HIF-1 in the induction of the EPO gene under hypoxia[104]. Likewise, Upstream stimulatory factor-2 (USF2) was reported to be specifically essential in the hypoxic induction of HIF-2 target genes including EPO in hepatic and renal cancer cell lines. USF2 activates HIF-2 target genes by binding to HIF-2 target gene promoters/enhancers, interacting physically and functionally with the N-TAD of HIF-2α and recruiting co-activators CBP and p300 to form a multi-factoral transcription complex on HIF-2 target gene promoters[105]. Signal transducer and activator of transcription-3 (STAT3) has been shown to be involved in the HIF-1 and activation of HIF-1 target genes during hypoxia in renal and breast cancer cell lines, but not HIF-2-mediated hypoxic transcriptional response. STAT3 physically and functionally interacts with the N-TAD of HIF-1α, but not with HIF-2α, and increases the recruitment of CBP and p300 co-activators to HIF-1 target promoters such as VEGF and haptoglobin promoters[106]. Steroid receptor coactivator-1 (SRC-1) has been shown to form a complex with CBP and interacts with the transactivation domains of HIF-1α. Thus, SRC-1 functions as a co-activator for HIF-1α and enhances HIF-1α hypoxia-inducible transactivation potential[107]. Apurinic/apyrimidinic endonuclease-1 (APE1), also known as Redox effector factor 1 (Ref-1), has been shown to act as a co-activator for HIF-1α through its redox activity, mediating the DNA-binding activity of HIF-1 and the expression of HIF-1 downstream genes, including VEGF and CA IX[23]. The enhanced expression of the Lactate dehydrogenase A (LDH-A) promoter in hypoxic conditions is dependent on two domains or sequences close to HIF-1 binding sites. One sequence is located in an analogous position to one of the crucial regions in the EPO 3′ hypoxic enhancer, and the other domain has the motif of a cAMP response element (CRE)[108]. Although all these domains are crucial for oxygen-regulated expression of LDH-A promoter, they are not capable of driving hypoxic induction in isolation[108]. In similar fashion, the induction of VEGF and EPO enhancers and hence synthesis are governed by the participation of CBP/p300–HIF-1/2 complexes[109]. In general, the induction of hypoxia-responsive genes is not solely dependent on HIF-1 recognition, but rather dependent on a tripartite array of sites participation[100]. Hypoxia activates the expression of Tyrosine hydroxylase (TH), the rate-limiting enzyme in the biosynthesis of dopamine, via increasing the rate of transcription of the TH gene and it is regulated by a region of the proximal promoter that contains a number of cis-acting regulatory elements including AP-1, Activating protein 2 (AP-2) and HIF-1[110, 111].

6. Targeting HIF-1/2 in cancer:

Taken together, the fact that solid tumors create their own characteristic hypoxic environment and the fact that hypoxia mediates the aggressive, metastatic and resistant forms of the tumors, it was rational to exploit the hypoxic tumor microenvironment to design and develop targeted therapies for cancers. Several approaches have been pursued including gene therapy, recombinant anaerobic bacteria, and the use of hypoxia-activated prodrugs[112, 113]. Another important route is to take advantage of the selective induction of the transcription factors HIF-1/2 under hypoxia. Ideally, selectivity could be achieved, and normal cells would remain unaffected. However, HIF-1/2 regulation pathways are highly complex and contain interconnected signaling cascades and overlapping mechanisms. This convolution manifests itself in two facts: 1) designing selective inhibitors of HIF-1/2 becomes highly challenging task, thus a growing number of HIF-1/2 targeting compounds that have been developed and classified based on their inhibitory mechanisms do not appear to inhibit the HIF-1/2 pathway as their specific target. 2) Considering, the regulatory mechanism of the transcriptional activation of HIF-1/2 which is dependent on a series of interrelated events, including elevated steady-state levels of HIF-1/2 α subunit via physiological stimulation as well as genetic alterations, HIF-1/2α nuclear translocation, heterodimerization with the β subunit, HREs-DNA binding, co-activators recruitment and formation of an active transcriptional complex, lots of efforts have been made to modulate and intervene each step of these HIF-1/2 regulating events. Many of the synthesized HIF-1/2 inhibitors act on HIF-1α, or HIF-2α, or both through direct mechanisms, while many other compounds and approved drugs have been shown to indirectly inhibit HIF-1/2 activity due to the connection between HIF-1/2 signaling and other cellular pathways such as the upstream pathways: the PI3K/AKT/mTOR pathways or the downstream cellular pathways: anti-VEGF-therapy. So far, many reviews on hypoxia- targeted anticancer agents have been reported[114, 115]. In this review, we describe a more updated set of new small molecule HIF inhibitors as potential anticancer agents listed in Table 1 and Figure 4.

Benzopyranyl 1,2,3-triazole (**1**) is a novel anticancer agent that was identified during in house chemical library screening and reported to work as HIF-1 inhibitor via increasing HIF-1α hydroxylation and subsequent ubiquitination and proteasomal degradation. It has an IC_{50} value of 2–24 nM in cancer cells. However, it decreases VEGF expression and angiogenesis in a dose-dependent manner. It has a synergistic effect with gefitinib, an Epidermal growth factor receptor (EGFR) inhibitor that is used clinically in lung and breast cancer with mutated and overactive EGFR, because the combination treatment inhibits tumor growth and angiogenesis in allograft model significantly[116].

BIX01294 (**2**) is a diazepinquinazolin-amine derivative that was originally identified as an Euchromatic histone-lysine N-methyltransferase 2 (EHMT2)/G9a inhibitor during a chemical library screening of small molecules[117]. EHMT2 is an essential enzyme that catalyzes the methylation of histone H3 at lysine residue 9 to form H3K9me2, which is an epigenetic marker[118, 119]. EHMT2 is highly expressed in human cancer cells such as in neuroblastoma and glioblastoma brain cancers and BIX01294 was reported to decrease the proliferation of neuroblastoma cells. BIX01294 was also reported to decrease HIF-1 expression in HepG2 human hepatocellular carcinoma cells via increasing the hydroxylation

of HIF-1α by increasing PHD2 and pVHL expressions and thus diminishing HIF-1α stability (at 1 μM range)[120]. However, it is noteworthy that G9a inhibition was reported to upregulate the HIF-1α and HIF-2α in breast cancer cells after using higher concentrations of BIX01294[121].

Cardenolides (**3**) were isolated from the latex and fruits of *Calotropis gigantea*, a medicinal plant native to Southeast Asia used traditionally for the treatment of several diseases including cancers. Cardenolides are steroids with 25–26 carbon atoms and a five or six-membered lactone ring at C-17. Among them, Calactin inhibits HIF-1 transcriptional activity with an IC_{50} value of 21.8 nM and it shows cytotoxic potency on human breast cancer cell line MCF-7 with an IC_{50} value of 45.2 nM[122].

CRLX-101(**4**) is a 20- to 30-nm diameter nanoparticle consisting of water-soluble cyclodextrins-based polymers that contain pendant carboxylate groups, camptothecin (CPT) and alternating repeat polyethylene glycol (PEG) blocks[123]. It was designed to accumulate into solid tumors and slowly release CPT in tumors over an extended period. In addition, it has the advantage of reducing the gastrointestinal (GI) toxicity related to CPT and improving patients' compliance due to its favorable safety profile[124]. It was demonstrated that CRLX-101 has improved the efficacy of the chemoradiotherapy for locally advanced rectal cancer and when use as monotherapy or in combination therapy[125]. However, CRLX101 showed improved efficacy when used alone or in combination therapy with Bevacizumab in metastatic triple negative breast cancer mouse models[126]. CPT, the active moiety in CRLX101, is a potent inhibitor of Topoisomerase I (Topo-I) and HIF-1α. It was originally identified as HIF-1 inhibitor in 2002 using a cell-based high-throughput screening of approximately 2000 compounds. It inhibits HIF-1α protein accumulation, hypoxic induction of VEGF mRNA and protein expression in hypoxic U251 human glioma cells in a dose-dependent manner. CRLX101 showed higher efficacy and good tolerability in preclinical mouse model of gastric adenocarcinoma compared to its parent compound CPT and its synthetic analogues Irinotecan (CPT-11) and Topotecan (TPT)[127]. Currently, CRLX101 is being evaluated in phase II clinical trials as in combination therapy with other anticancer drugs for several tumor types[124]. The established dose for CRLX101 concluded from phase I clinical trial is 15 mg/m^2 to be given intravenously every 2 weeks[124]. The preferential safety profile of CRLX101 was also confirmed in a pilot trial using the same recommended dose in patients with chemotherapy-refractory gastroesophageal cancer[125, 128].

EZN-2208 (**PEG-SN38**, **5**) is the multiarmed PEG backbone linked form of SN38. SN38 (7-ethyl-10-hydroxy-camptothecin) is the active metabolite of CPT-11 and it has anti Topo-I activity[129]. EZN-2208 was made to address the poor solubility of SN38 and it has the same anticancer potency as the native SN38 compound. Furthermore, it showed significantly greater antitumor activity than CPT-11 in several human tumor xenograft models including a CPT-111 resistant model[130]. EZN-2208 reduced the expression and transcriptional activity of HIF-1α in preclinical models[131]. Moreover, EZN-2208 in combination therapy with All-trans retinoic acid-arsenic trioxide (ATRA-ATO), the current standard of care for patients with acute promyelocytic leukemia (APL), was highly effective in treating patients with APL who develop resistance to ATO or patients carrying the PLZF-RARα fusion protein[132]. This synergistic effect was foreseeable since PML-RARα, the oncogenic fusion proteins

found in 95% of the (APL) cases, behave as transcriptional co-activators of HIF-1α in leukemic promyelocytes of APL patients, and HIF-1α regulates leukemia-initiating cells (LICs) maintenance and leukemia progression[133]. In addition, ATRA increases HIF-1α levels in APL cells via induction of the transcriptional activation of PML-RARα[134]

Glyceollins (a mixture of glyceollin I, II, and III, **6**) are a group of phytoalexins that are synthesized de novo in soybean via the stimulatory action of elicitors as a protective mechanism against microbial invasion, ultraviolet radiation, and chemical stressors[135, 136]. Glyceollins exhibit various biological functions including anti-estrogenic activity[137], anti-contractile activity in vascular smooth muscle[138], enhanced insulin sensitivity[139] and anti-melanin synthesis activity[140]. Most importantly, Glyceollins regulate tumor growth and inhibit the expression of VEGF in cancer cells through regulation of HIF-1α[141, 142]. They perform their HIF-1 inhibitory action by two means: 1) they block HIF-1α translation via inhibiting the PI3K/AKT/mTOR pathway under hypoxic conditions. 2) They interfere with Hsp90 binding activity and thus decrease HIF-1α stability.

IDF-11774 (**7**) is another aryloxyacetylaminobenzoic acid analogue like LW6, which have been reported to inhibit the accumulation of HIF-1α via regulation of VHL expression[143]. LW6 was discovered based on a high-throughput cell-based reporter assay for in house chemical library in human hepatocellular carcinoma Hep3B cells[144]. IDF-11774 is an (E)-phenoxyacrylic amide derivative of LW6 where the oxyacetylamide linker has been replaced with an oxyacrylic amide to provide a more constrained conformation. In comparison to LW6, IDF-11774 showed superior potency in colorectal carcinoma HCT116 cells and improved aqueous solubility. IDF-11774 promotes HIF-1α degradation and inhibits its accumulation in colorectal cancer cells *in vitro* and *in vivo*[145, 146]. IDF-11774 was shown to significantly inhibit mitochondrial respiration and increase the intracellular oxygen tension, and thus promote the proteasomal degradation of HIF-1α[146]. Intriguingly, IDF-11774 was reported to act as Hsp70 inhibitor by binding to the allosteric pocket of Hsp70[147]. Moreover, IDF-11774 suppresses the hypoxia-induced mRNA expression of hypoxia target genes including VEGF and EPO[145]. Currently, IDF-11774 is being evaluated in phase I clinical trial for cancer therapy by the Korean Food and Drug Administration (KFDA)[146].

LBH589, also known as (**panobinostat**, **8**), is a pan histone deacetylase (HDAC) inhibitor that exhibits antitumor effects *in vitro* and *in vivo* in various hematologic malignancies and solid tumors, including diffuse large B-cell lymphoma, Hodgkin lymphoma, multiple myeloma, hepatocellular carcinoma, pancreatic cancer and non-small cell lung cancer[148–151]. Moreover, LBH589 showed anticancer activity against glioblastoma both *in vitro* and *in vivo* through interfering with HIF-1α stability and inducing its degradation. LBH589 disrupts Hsp90/Histone deacetylase 6 (HDAC6) complex because Hsp90 chaperone activity is regulated by its interaction and reversible acetylation by HDAC6 and inactivation of HDAC6 leads to Hsp90 hyperacetylation, its dissociation from p23 co-chaperone, and a loss of Hsp90 activity[152]. However, the newly synthesized HIF-1α molecules need to interact with the chaperone Hsp90 to complete its maturation and LBH589 treatment causes disruption of Hsp90-mediated folding of HIF-1α leading to its subsequent degradation by proteasome[153, 154] However, LBH589 attenuates hypoxia in a much higher level of complexity due to its influence on multiple HDACs. For instance, in

virtue of its pan inhibitory activity LBH589 could disrupt HIF-1α stability via inhibiting HDAC 1, 3, and 4 because they reported to bind directly to the ODDD of HIF-1α and induce deacetylation of lysine residues, and thus invoke HIF-1α stability and HIF-1 transactivation function in hypoxic conditions[155, 156]. Moreover, LBH589 could simultaneously target Histone deacetylase 7 (HDAC7) since HDAC7 was reported to form a complex with HIF-1α and p300 after co-translocation to the nucleus under hypoxic conditions and thus increases the transcriptional activity of HIF-1α[157]. However, it worthy to mention that a recently reported phase II clinical trial of combined LBH589 with Bevacizumab in patients with recurrent high-grade glioma (HGG) did not significantly improve the 6-month progression-free survival (PFS6) compared with historical controls of Bevacizumab monotherapy[158].

MPT0G157 (**9**) an indole-3-ethylsulfamoylphenylacrylamide compound was developed based on the core structure of PXD101 (Belinostat) and LBH589, HDAC inhibitors[159]. MPT0G157 exerted potent inhibition against HDAC1, 2, 3 and 6 and subsequently resulted in hyper-acetylation of Hsp90 and of HIF-1α, leading to its subsequent degradation. In addition to its anti-inflammatory effect, MPT0G157 showed anticancer activity *in vitro* and *in vivo* particularly in human colorectal cancer (HCT116) cells[160].

Vorinostat also known as (Suberoylanilide Hydroxamic Acid/SAHA, **10**) is an FDA approved HDAC pan inhibitor used clinically for the treatment of cutaneous T cell lymphoma (CTCL). It displayed anti HIF-1/2 activity in hepatocellular carcinoma (HCC) (both *in vitro* and *in vivo*), osteosarcoma (OS), and glioblastoma (GBM) cell lines *in vitro*[161]. Different hypotheses are proposed to elucidate the suppression of hypoxia signaling mediated by Vorinostat. Although SAHA was shown to inhibit HIF-1/2 stabilization via direct acetylation of Hsp90 and increase HIF-1/2 degradation through a ubiquitin-dependent mechanism, other reports support that SAHA and other class I and II HDAC inhibitors enhance HIF-1/Hsp70 interactions and mediated HIF-1α degradation via non-ubiquitin-mediated degradation by the 20S proteasome[162]. Moreover, Vorinostat inhibits the interaction between HIF-1/2α and Importin and hence blocks HIF-1/2 α nuclear translocation[161]. However, Hutt *et al* have demonstrated that Vorinostat inhibits HIF-1α translation through Histone deacetylase 9 (HDAC9) and indirectly interferes with Eukaryotic translation initiation factor 3subunit G (eIF3G) in hepatocellular carcinoma[163].

NNC 55–0396 (**11**) is a derivative of Mibefradil and it is a selective T-type Ca2+ channel blocker. It was identified based on a library screening study and cell growth assay with glucose or galactose medium, where it exhibited stronger inhibitory effects on cell growth in galactose medium than in glucose, an indication of modulating the mitochondrial function. NNC 55–0396 was found to significantly suppress the hypoxia-induced mitochondrial ROS generation and thus blocks HIF-1 activation and tumor growth and angiogenesis *in vitro* and *in vivo*[164]. Increased mitochondrial ROS generation in hypoxic cells triggers the HIF-1 induction which in turn activates the transcription of the genes encoding Pyruvate dehydrogenase kinase 1 (PDK1) and LDH-A in order to decrease the production of the ROS. This represents a pivotal regulating mechanism that functions to prevent excessive ROS production[165]. By the virtue of inducing PDK1 and LDH-A, HIF-1 can limit the delivery of the reducing equivalents NADH and FADH2 to the electron-transport chain, thus blocks the

mitochondrial respiration. In addition, NNC 55–0396 increased the hydroxylation of HIF-1α and its subsequent ubiquitination and degradation. Moreover, NNC 55–0396 suppressed the de novo HIF-1 α synthesis via inhibition the phosphorylation of mTOR and p70S6K.

Kresoxim-methyl analogues (**12**) were designed and showed to inhibit hypoxia-induced HIF-1 transcriptional activation with IC_{50} values of 0.60–0.94 μM in human colorectal cancer (HCT116) cells. They work by increasing the intracellular oxygen tension under hypoxic condition, and thus promoting the ubiquitin-dependent proteasomal degradation of HIF-1α and impairing its accumulation[166]. Kresoxim-methyl is marketed synthetic analogue of strobilurin fungicide with the representative toxophore methyl methoxy-imino-acetate. It is a mitochondrial inhibitor specifically targets the cytochrome bc1 complex (complex III) of the mitochondrial electron transport chain by binding to its Q_0 site, therefore; perturbs the ROS mitochondrial production responsible for hypoxia-dependent stabilization of HIF-1.

PT2385 (**13**) and **PT2399** (**14**) are selective HIF-2α inhibitors that allosterically blocks its dimerization with HIF-1β. They are reported to have efficacy in clear cell renal cell carcinomas (ccRCC) cell lines and tumor xenografts. However, they exhibited preferable safety profile in xenograft models, with no adverse cardiovascular side effects, which have been associated with anti-VEGF receptor chemotherapeutic agents. They were identified using structure-based drug design using the PAS-B domains of HIF-2α and ARNT, and it was proved that they bind to the internal cavity of the HIF-2α PAS-B domain[167]. However, phase I clinical trial demonstrated that PT2385 has a favorable safety profile and is active in patients with heavily pretreated ccRCC[168, 169].

7. Conclusion:

From the date of HIF-1 discovery by Semena and co-workers[170], and from the first published report by Zhong *et al*[171]that linked HIF-1α overexpression in primary human cancers and their metastases, and this field has since expanded into a major area of cancer research. This partially due to the large number of clinical data that demonstrate an association between increased levels of HIF-1/2α proteins with increased both radiotherapy and chemotherapy resistance, cancer progression and patient mortality in many different human cancers. Beside intratumoral hypoxia, diverse sets of mechanisms have been reported to contribute to HIF-1/2 signaling and regulations, including, low–molecular weight signaling molecules such as ROS, cytokines and growth factors, tumor suppressor loss of function and oncogene gain of function. This complexity in the regulation pathways has made the process of rational design of HIF-1/2 α inhibitors very challenging. A growing number of HIF-1/2 α inhibitors have been identified, although; no selective HIF-1/2 α inhibitor has been clinically approved. In addition, several approved drugs have been reported to indirectly affect the HIF-1/2 α pathway, which reflects the extended connectivity between HIF-1/2 signaling and other cellular pathways. Therefore, targeting these pathways in cancer leads to secondary (indirect) inhibition of HIF activity. Future directions would be directed towards developing selective HIF-1/2 α inhibitors and addressing those combinations of drugs to which addition of a HIF-1/2 inhibitor will have additive or synergistic effects.

8. Expert opinion:

The reported literature regarding hypoxia and its playmakers HIFs provide a good insight into the complexity that characterize HIFs regulation pathways. The stability and degradation of HIF-1α is differentially affected by short or prolonged exposure to hypoxia, and the cross talks between HIF-1/2 and its affecter genes such as autocrine growth factors and other cellular stress response mediators are complicated. However, further research is needed to unravel the extensive complexity of HIF-1/2 regulation and to develop a more precise anticancer treatment. Many types of cancer such as breast, brain, cervical, colorectal, acute lymphoid, myeloid leukemias, melanoma, gastric cancer as well as liver, lung, ovarian, and pancreatic have been shown to respond poorly to chemotherapeutic agents and radiation therapy because of increased HIF-1/2 activation. HIF-1/2 activation contributes to cancer patients' mortality through activation of genes encoding proteins that mediate vascularization, immune evasion, metabolic reprogramming, growth factor signaling, invasion, tumor progression, and metastasis. Consequently, there have been great interests in developing inhibitors targeting HIF-1/2. Even though there are large number of chemical compounds and approved drugs that have been shown to inhibit HIF-1/2 activation via a variety of molecular mechanisms, HIF-1/2 inhibition selectivity is far from fulfillment. This makes developing selective HIF-1/2 inhibitors as the ultimate goal for this area of research. Recurrent and metastatic triple-negative breast cancer tumors where paclitaxel or carboplatin is the established therapy are associated with overexpression of HIF-1α mRNA and protein and HIF-1 target genes. Consistently, co-administration of a HIF-1 inhibitor counteracts these negative pathological responses[172]. The addition of HIF-1/2 inhibitors to the current treatment have been proven advantageous in a wide range of preclinical studies including, acute and chronic myeloid leukemia, glioblastoma, colon, and liver cancer. Taken together, it is reasonable to argue that adding HIF-1/2 inhibitors to the cancer treatment regimens is a necessity. Ideally, the combination therapy using two or more chemotherapeutic agents should exert additive or synergistic effects and could preferably include multiple drugs that inhibit HIF-1/2 activity by different molecular mechanisms for long-term efficacy. For instances, large number of ccRCC show genetic mutation in VHL and TCEB1, which encodes Elongin C. These defects impair the classical regulation pathway of HIF-1/2, cause failure to degrade HIF-α subunits and result in its accumulation under normoxic conditions[173]. Furthermore, inhibition of HIF-2α has proven to be therapeutically useful in in mice bearing pVHL-defective clear-cell renal carcinoma[174], and HIF-2α knockdown studies phenocopy the effects of pVHL reintroduction with respect to decreased expression of hypoxia-inducible genes and tumor growth suppression[175]. In contrast, HIF-1α is often deleted or mutated in a subtype of ccRCC characterized by chromosome 14q loss of function because HIF-1α gene (*HIF1A*) resides in this chromosome[176]. These data along with others suggest that HIF-2α might be the pathogenic driver in ccRCC and underscore the therapeutic potential of HIF-2α inhibitor for ccRCC treatment. PT2385 may particularly effective in the treatment of renal cell carcinomas especially with the positive results of its phase I clinical trial. However, PT2399 prolonged treatment causes cross-resistance in xenograft models, probably due to mutations in the binding site in HIF-2α[177]. Thus, co-administration of multiple drugs that inhibit HIF-2 activity by different molecular mechanisms may be necessary. However, drug-drug interaction, side effects and toxicity

should be considered upon choosing the HIF-1/2 inhibitor as some of the currently available pan-HIF-1/2 inhibitors such as echinomycin and YC-1 have dose-limiting side effects. Overall, while it is still very challenging to develop more specific and thus selective HIF-1/2 inhibitors, with increased understanding of the HIFs structures, molecular mechanisms, and interactions with other signal transduction pathways, we certainly are seeing more and more lights in the tunnel for developing more efficacious HIF-1/2 inhibitors in the near future.

Funding:

This research is supported by National Institutes of Health/National Cancer Institute grant R01CA193609 (to W Li). The content is solely the responsibility of the authors and does not necessarily represent the official views of the NIH/NCI.

Reviewer Disclosures

One referee has received research funding and has consulted for Peloton. Peer reviewers on this manuscript have no other relevant financial relationships or otherwise to disclose.

List of Abbreviations used:

4E-BP1	eIF4E-binding protein 1
AP-1	Activator Protein-1
AP-2	Activating protein 2
APL	Acute promyelocytic leukemia
ATRA-ATO	All-trans retinoic acid-arsenic trioxide
APE1	Apurinic/apyrimidinic endonuclease-1
AhR	Aryl hydrocarbon receptor
ARD1	Arrest defective-1
ARNT	Aryl hydrocarbon receptor nuclear translocator
ATM	Ataxia telangiectasia mutated
CPT	Camptothecin
CA IX	Carbonic anhydrase-9
CHIP	Carboxyl terminus of Hsp70-interacting protein
CK1δ	Casein kinase 1delta
CRM1	Chromosomal maintenance 1
CRE	cAMP response element
ccRCC	Clear cell renal cell carcinomas
COP9	Constitutive photomorphogenic-9

CSN5	signalosome subunit 5
CUL2	Cullin scaffold protein
CTCL	Cutaneous T cell lymphoma
CDK1	Cyclin-dependent kinase 1
EGF	Epidermal growth factor
EHMT2	Euchromatic histone-lysine N-methyltransferase 2
eIF3G	Eukaryotic translation initiation factor 3subunit G
eIF-4E	Eukaryotic translation initiation factor 4E
EPAS1	Endothelial PAS domain protein 1
EPO	Erythropoietin
ET-1	Endothelin-1
ENO1	Enolasen1
EGFR	Epidermal growth factor receptor
XPO1	Exportin 1
FGF-2	Fibroblast growth factor 2
FIH	Factor inhibiting HIF
FRAP	Rapamycin-associated protein
GATA-2	GATA-binding factor 2
GBM	Glioblastoma
GLUT-1	Glucose transport-1
GLUT-3	Glucose transport-3
GSK3β	Glycogen synthase kinase 3β
HAF	Hypoxia-associated factor
HNF4	Hepatocyte nuclear factor-4
HCC	Hepatocellular carcinoma
HGG	High-grade glioma
HDAC	Histone deacetylase
HDAC1	Histone deacetylase 1
HDAC6	Histone deacetylase 6

HDAC7	Histone deacetylase 7
HDAC9	Histone deacetylase 9
HIFs	Hypoxia- inducible factors
HIF-1	Hypoxia-inducible factor-1
HIF-2	Hypoxia-inducible factor-2
HIF-3	Hypoxia-inducible factor-3
HIF-1α	Hypoxia-inducible factor-1 α subunit
HIF-2α	Hypoxia-inducible factor-2 α subunit
HIF-3α	Hypoxia-inducible factor-3 α subunit
HIF-1β	Hypoxia-inducible factor-1 β subunit
HIF1A	HIF-1α gene
HLF	HIF-1α-like factor
HREs	Hypoxia-responsive elements
HRF	HIF-1α related factor
Hsp70	Heat shock protein 70
Hsp90	Heat shock protein 90
IGF-1	Insulin-like growth factor-1
IGF-2	Insulin-like growth factor-2
IPAS	Inhibitory PAS domain protein
CPT-11	Irinotecan
Jab1	Jun activating binding protein 1
LDH-A	Lactate dehydrogenase A
MAPK/ERK	Mitogen-activated protein kinase
Mdm2	Murine double minute 2
MNK1	MAP kinase interacting kinase 1
MNK2	MAP kinase interacting kinase 2
MOP-1	Member of the PAS superfamily-1
MTA1	Metastasis-associated protein 1
MTA2	Metastasis-associated protein 2

mTOR	Mammalian target of rapamycin
mTORC1	mTOR complex 1
mTORC2	mTOR complex 2
NEPAS	Neonatal and embryonic PAS
NF-1	Nuclear Factor-1
OS	Osteosarcoma
PCAF	CBP/p300-associated factor
p70S6K	p70 ribosomal protein S6 kinase
PI3K	Phosphatidylinositol 3- kinase
PHD 1–4	Prolyl hydroxylases domain enzymes 1–4
PKA	Protein kinase A
AKT	Protein kinase B
PLK3	Polo-like kinase 3
PDK1	Pyruvate dehydrogenase kinase 1
PEG	polyethylene glycol
ROS	Reactive oxygen species
RACK1	Receptor of activated protein kinase C 1
Ref-1	Redox effector factor 1
rpS6	Ribosomal protein S6
STAT3	Signal transducer and activator of transcription-3
SIRT1	Sirtuin 1
SRC-1	Steroid receptor coactivator-1
SAHA	Suberoylanilide hydroxamic acid
TH	Tyrosine hydroxylase
Topo-I	Topoisomerase I
TPT	Topotecan
USF2	Upstream stimulatory factor-2
VEGF	Vascular endothelial growth factor
pVHL	von Hipple-Lindau

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Article Highlights:

- Solid tumors develop hypoxia because of deficiency or abolishment in oxygen supply in the tumor microenvironment. Hypoxia has negative impacts on radiotherapy and chemotherapy, and it potentiates tumor metastasis, genomic instability, and poor prognosis.
- Hypoxia induces short and long-term responses in hypoxia-responsive elements bearing genes through its transcriptional factors HIFs, heterodimeric proteins that consist of two proteins, HIF-α and HIF-β. HIF-α stability is the primary determinant for the regulation of HIF activity. HIF-α has three closely related homologues, HIF-1α, HIF-2α and HIF-3α.
- In normoxia, HIF-α is regulated by O_2-dependent prolyl hydroxylation which facilitates its ubiquitylation by E3 ubiquitin-protein ligases containing the von Hippel–Lindau. Thereafter, the ubiquitinated HIF-α is signaled for proteasomal degradation. HIF-1/2α is overexpressed in human cancers as a result of intratumoral hypoxia, low–molecular weight signaling molecules such as reactive oxygen species, gain-of-function mutations in oncogenes and loss-of-function mutations in tumor-suppressor genes. HIF-1/2 regulation pathway is highly complex and contains interconnected signaling cascades and overlapping mechanisms.
- The regulatory mechanism of the transcriptional activation of HIF-1/2 is dependent on a series of interrelated events, which includes elevated steady-state levels of HIF-α subunit, localization of HIF-α inside the nucleus, heterodimerization with the β subunit, hypoxia-responsive elements-DNA binding, increased transcriptional activity mediated by C-TAD and N-TAD of HIFα, as well as co-activators recruitments.
- HIF-1/2α overexpression is linked with primary human cancers and their metastases, radiotherapy and chemotherapy resistance, and patient mortality. In preclinical and clinical studies, inhibition of HIF-1/2 activity has marked effects on angiogenesis and tumor growth. Thus, efforts are directed towards developing selective HIF-1/2 inhibitors.

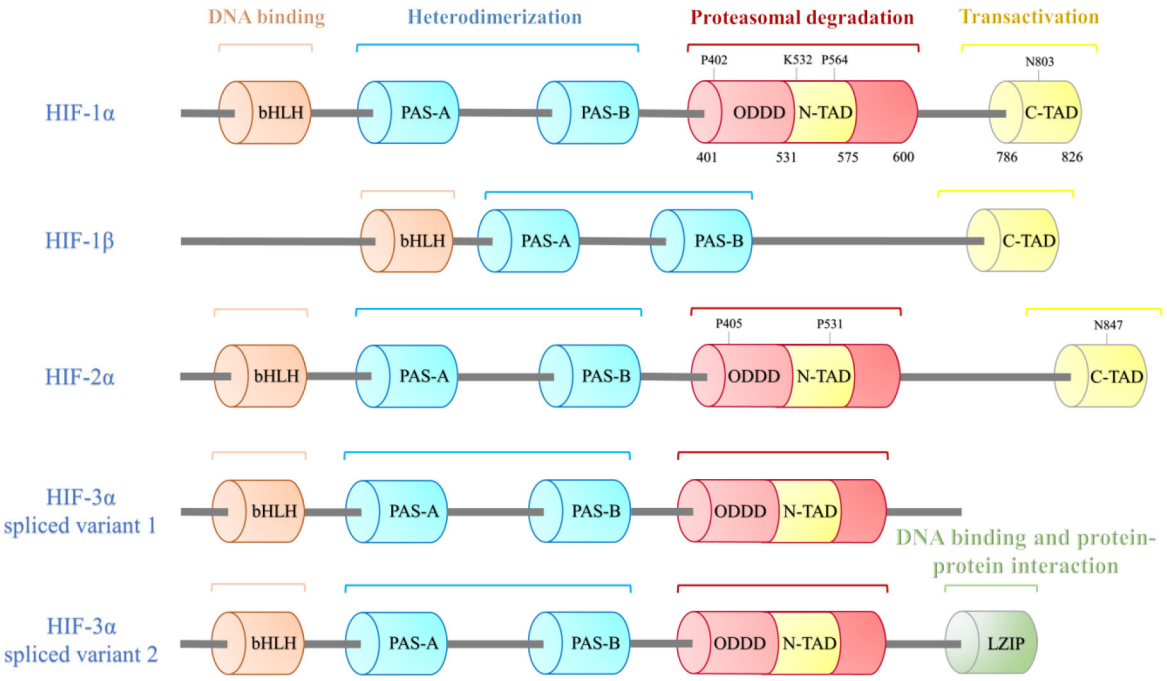

Figure 1.

Functional domain structures of HIF isoforms and their potential function. Columns represent different function domains. The hydroxylation sites are shown above the domain. HIF isoforms are bHLH–PAS proteins, they all have a bHLH motif, two PAS domains (PAS-A and PAS-B) for the heterodimerization between HIF-α and HIF-1β. Unlike HIF-1β, HIF-α subunits have an ODDD that mediates hydroxylation of two proline (P) residues and the acetylation of a lysine (K) followed by proteasomal degradation, a N-TAD within the ODDD and a C-TAD, which involved in transcriptional activation. The proline residues are conserved in HIF-1/2α subunits. Multiple HIF-3α splice variants exist, such as HIF-3α variant 1 without C-TAD and HIF-3α variant 2 with a LZIP, which mediates DNA binding and protein-protein interaction.

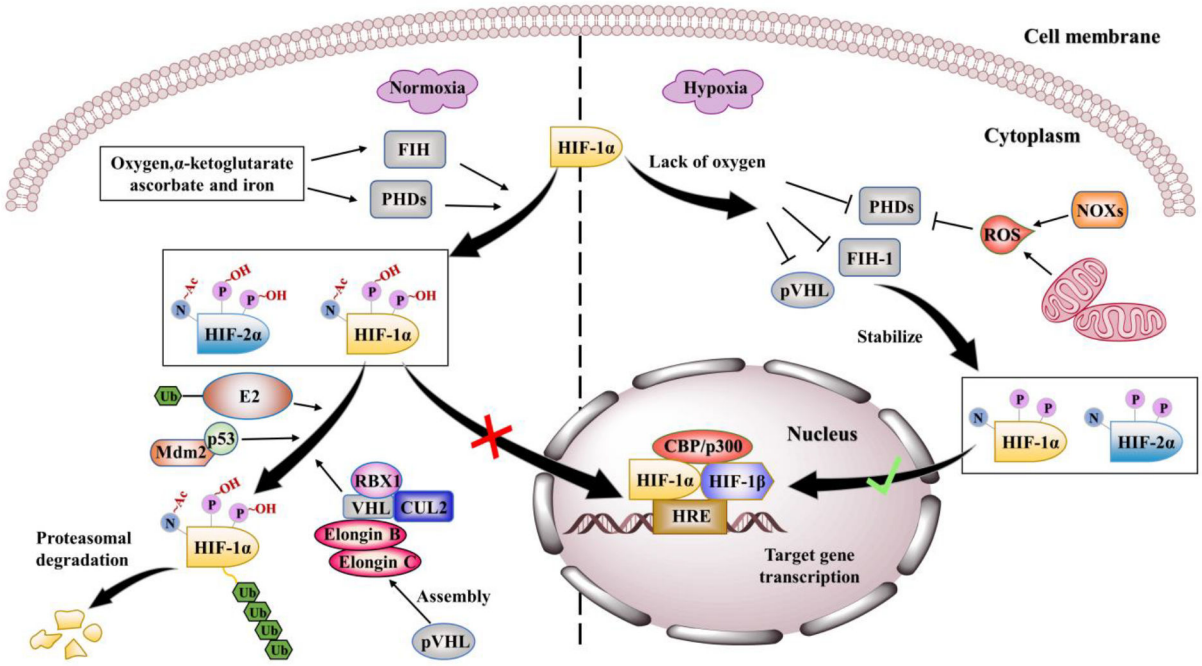

Figure 2.

Schematic diagram of canonical mechanisms regulating HIF-1/2. Under normoxic conditions (left panel), PHDs and FIH hydroxylate HIF-1/2α on proline residues and asparagine residue and trigger formation of hydroxylated HIF-1/2α. In the meantime, pVHL mediates the assembly of a complex containing VHL, Elongin B, Elongin C, CUL2 and RBX1, which binds ubiquitin-conjugated E2 component to accomplish ubiquitination of HIF-1/2α proteins. P53 is able to recruit an E3 ubiquitin–protein ligase, Mdm2, to help the proteasomal degradation of HIF-1α mediated by an E2 and E3 ubiquitin ligase–pVHL complex. Besides, hydroxylation of the asparagine residue in the C-TAD of HIF-1/2, FIH blocks the essential interaction between HIF-1α and co-activators such as CBP/p300. However, under hypoxic conditions (right panel), pVHL, PHDs and FIH activities are inhibited by limited oxygen, and ROS generation mechanisms in mitochondria and others such as NADPH oxidases (NOXs), leading to the escape of HIF-1/2α from proteasomal degradation. Thus, the HIF transcriptional complex binds to the HREs motif on the DNA of target genes and activates the target gene transcription.

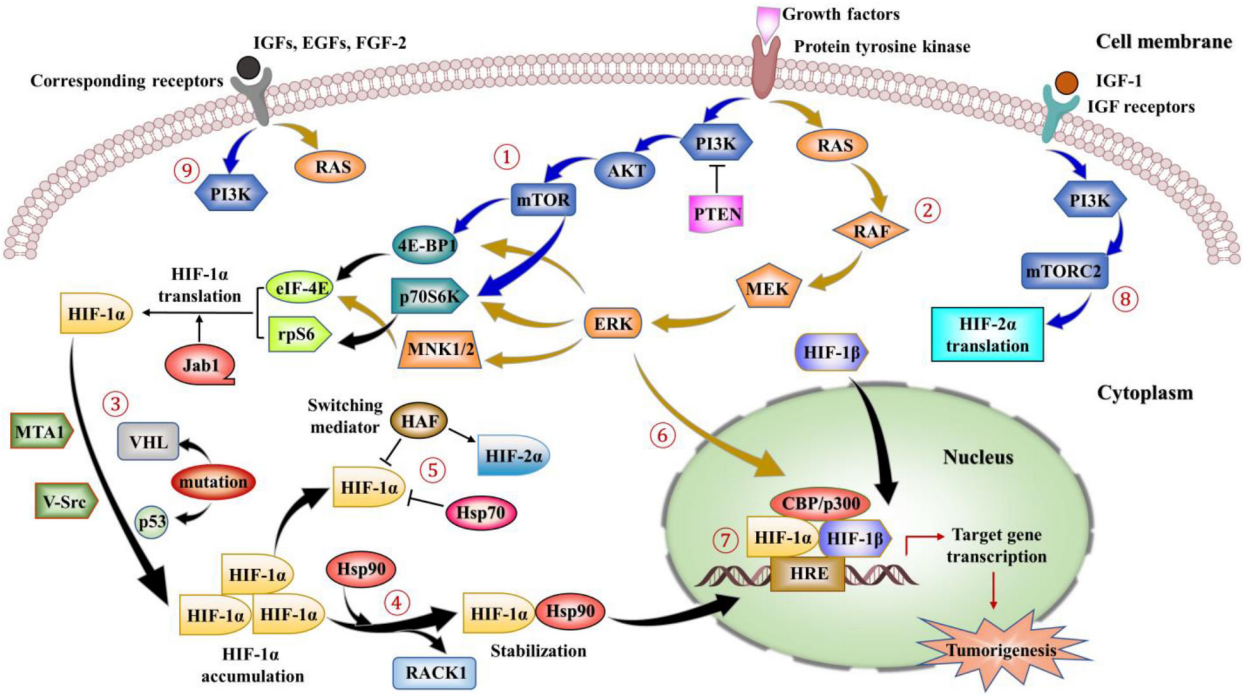

Figure 3.

Main signaling involved in non-canonical pathways regulating HIF-1/2. ①-②: HIF-1α regulation could be initiated by growth factors stimulation via activation of protein tyrosine kinases (PTKs). This stimulation leads to downstream signaling activations via the PI3K–AKT–mTOR pathway (indicated by the blue arrows) and MAPK/ERK pathway (indicated by dark yellow arrows). mTOR and ERK signaling further mediate phosphorylation and activation of three downstream effectors, 4E-BP1, p70S6K and MNK1/2, followed by the activation of eIF-4E and rpS6. Finally, these phosphorylation actions result in enhanced translation of HIF-1α mRNA into protein. In addition, Jab1 may promote the transcriptional activity of HIF-1α, while PTEN, a negative regulator of the PI3K, could downregulate the HIF-1α expression. ③: Mutations of VHL, PTEN and p53 result in increased expression of HIF-1α, as well as activations of oncogenes, such as MTA1 and V-Src. ④: Hsp90 competes with the RACK1 for binding to the HIF-1α PAS domain since RACK1 destabilizes HIF-1α via proteasomal degradation pathway. HIF-1α further accumulates in the cytoplasm with the help of Hsp90 which assists the protein folding, prevents the degradation of HIF-1α by the proteasome and contributes its nuclear translocation. ⑤: HIF-1α is also regulated by HAF by inducing the degradation of HIF-1α, while increasing HIF-2α transactivation. Compared with Hsp90, Hsp70 could mediate HIF-1α degradation in the prolonged hypoxia but not HIF-2α. ⑥: ERK phosphorylates the co-activator CBP/p300 and increases HIF-1α/p300 complex formation. ⑦: In the nucleus, the HIF complex binds to the HREs motif on the DNA of target genes and activates the transcription, causing the upregulation of genes involved in cell proliferation, cell survival, angiogenesis and tumorigenesis. ⑧: IGF-1 activation could induce the transcription of HIF-2α via PI3K-mTORC2 system. ⑨: Stimulations of IGFs, EGFs and FGF-2 on corresponding receptors induce HIF-1α

expression and protein synthesis through activation of both PI3K/AKT and MAPK pathways.

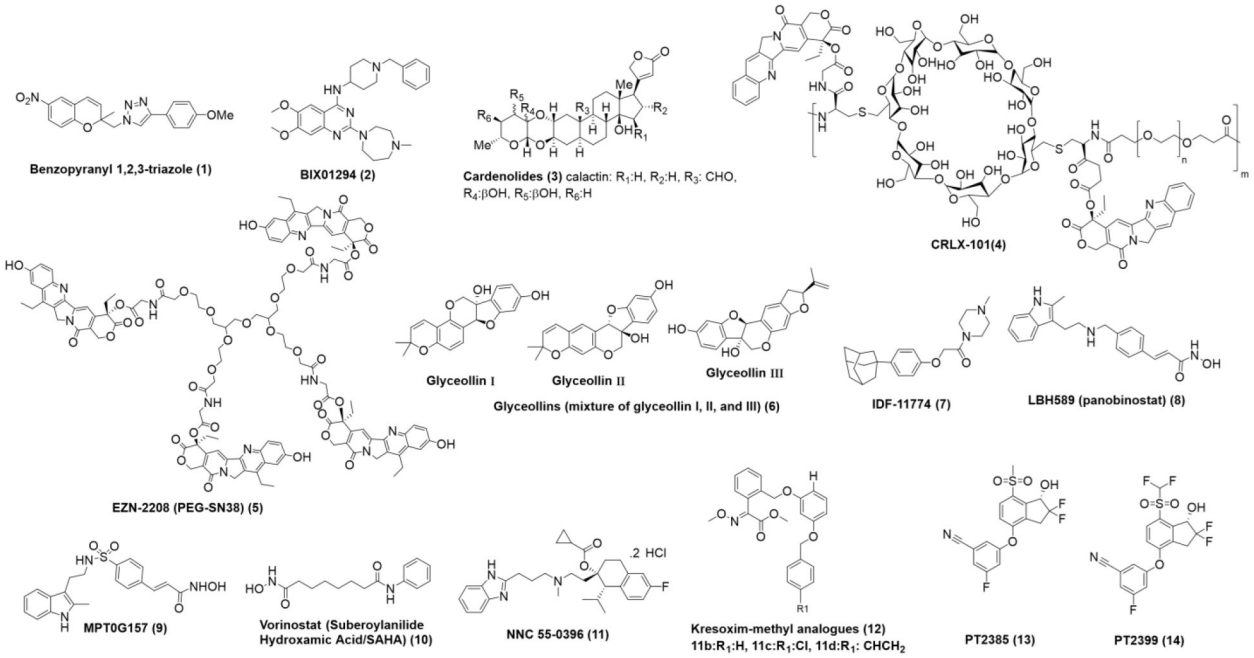

Figure 4.
Recently reported chemical structures of molecules inhibiting HIF-1/2.

Table 1.

Newly reported HIF inhibitors

Inhibitor	Mechanism of Inhibition
Benzopyranyl triazole (**1**)	Increased HIF-1α hydroxylation
BIX01294 (**2**)	Increased HIF-1α hydroxylation/HIF-1α protein degradation
Cardenolides (**3**)	Decreased HIF-1 transcriptional activity
CRLX-101 (**4**)	Decreased HIF-1α protein accumulation
EZN-2208 (**5**)	Decreased HIF-1α protein expression and transcriptional activity
Glyceollins (**6**)	Decreased HIF-1α translation/stability
IDF-1174 (**7**)	Increased HIF-1α degradation
LBH589 (**8**)	Increased HIF-1α degradation
MPT0G157 (**9**)	Increased HIF-1α degradation
Vorinostat (**10**)	Decreased HIF-1/2α translation/nuclear localization/stability
NNC 55–0396 (**11**)	Decreased HIF-1α protein expression/translation/Increased HIF-1α hydroxylation
Kresoxim-methyl analogues (**12**)	Decreased HIF-1α stability
PT2385 (**13**) and PT2399 (**14**)	Decreased HIF-2 heterodimerization