Hypoxia inducible factor pathway inhibitors as anticancer therapeutics

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Abstract

Hypoxia is a significant feature of solid tumor cancers. Hypoxia leads to a more malignant phenotype that is resistant to chemotherapy and radiation, is more invasive and has greater metastatic potential. Hypoxia activates the hypoxia inducible factor (HIF) pathway, which mediates the biological effects of hypoxia in tissues. The HIF complex acts as a transcription factor for many genes that increase tumor survival and proliferation. To date, many HIF pathway inhibitors indirectly affect HIF but there have been no clinically approved direct HIF inhibitors. This can be attributed to the complexity of the HIF pathway, as well as to the challenges of inhibiting protein–protein interactions.

Hypoxia in cancer

Cancer is the second most common cause of death in the USA, with practically one in every four deaths due to cancer [301]. Moreover, the most recent data report that in 2008 there were over 12.5 million new cases of cancer diagnosed worldwide and that by 2030 this number is anticipated to swell to over 20 million [302]. Many cancers involve solid tumor formation, and during periods of rapid growth, tumors out-grow existing blood supply, leading to the development of hypoxic (partial oxygen pressure of less than 5 Torr) and anoxic regions [1]. Tumors remedy this by producing angiogenic factors that lead to the formation of tumor vasculature, although, with structural and functional anomalies. These include arteriovenous shunts, blind ends, occlusions, high angle branching patterns, and broken, leaky vessels [2]. Abnormalities in the tumor vasculature limit oxygen delivery, leading to acute hypoxia [3].

Hypoxic tumors are more resistant to radiation and chemotherapy, are more invasive, are genetically unstable, resist apoptosis and have greater metastatic potential; all of which leads...
to poorer prognosis overall for patients [4,5]. It has been demonstrated that tumor irradiation is three-times more effective when carried out under oxygen-rich versus anoxic conditions. Moreover, the effectiveness of anticancer therapeutics that target rapidly dividing cells is reduced against hypoxic cells due to the decreased rate of cell proliferation that increases with distance from vasculature [6]. Another factor in treatment resistance is due to the cancer stem-like cells (CSCs), which are a relatively rare subpopulation of tumor cells with self-renewal capacity. CSCs reside in hypoxic niches of tumors and are more resistant to radio- and chemo-therapy-induced DNA damage, allowing them to survive the treatment and repopulate the tumor with their progeny. Typically, prolonged hypoxia triggers cell apoptosis, but in tumors, it can lead to the selection of tumor cells with mutant p53, which are resistant to apoptosis and confer a more malignant phenotype [7]. Clinical studies have shown that many cancers with hypoxic tumors are more likely to be metastatic, including soft tissue sarcomas, squamous cell head and neck carcinomas, cervical carcinomas and malignant melanomas [8]. Therefore, hypoxia can either lead to cell death through apoptotic or necrotic pathways, or to cell proliferation through activation of various other pathways [3].

The hypoxia inducible factor pathway

The hypoxia inducible factor (HIF) pathway is a major mediator of the biological effects of hypoxia in tissues (Figure 1) [9]. HIFs are basic-loop-helix-loop motif heterodimeric transcription factors composed of two subunits: an oxygen-regulated \( \alpha \) subunit (HIF-1\( \alpha \), HIF-2\( \alpha \), or -3\( \alpha \)) and the constitutively expressed HIF-1\( \beta \) (also called aryl hydrocarbon receptor nuclear translocator) [10]. Under normoxic conditions, HIF-\( \alpha \) subunits are hydroxylated by \( \text{Fe}^{2+} \)-dependent HIF prolyl hydroxylases (PHD), mostly PHD2, at two proline residues (402 and 564 in human HIF-1\( \alpha \)) located in the oxygen-dependent degradation domain [11,12]. This dihydroxylated form of HIF-\( \alpha \) is bound by VHL, which is an E3 ubiquitin ligase that leads to the ubiquitination of HIF-\( \alpha \) and subsequent proteasomal degradation [13,14]. This interaction between VHL and HIF-1\( \alpha \) has been shown in some instances to be promoted by acetylation of HIF by the ARD1 acetyltransferase at lysine 532, increasing ubiquitination and degradation [15]. However, the role of acetylation of HIF is still somewhat controversial and needs further exploration.

Conversely, under hypoxic conditions, PHDs lose activity and HIF-\( \alpha \) units are no longer degraded. As HIF-1\( \alpha \) or HIF-2\( \alpha \) accumulate, they bind to HIF1-\( \beta \) and form the HIF heterodimers, which translocate to the nucleus and, along with co-activators (p300 and CBP), form the transcriptional complexes that bind to hypoxic response elements (HREs) in the regulatory regions of many genes [16]. HREs are composite cis-acting elements, comprising the necessary but not sufficient HIF-binding site with the consensus sequence RCGTG (where R = purine A or G) and a HIF-ancillary sequence [17]. The trans activation potential of the HIF-\( \alpha \)/p300 complexes is regulated via deacetylation of p300 by histone deacetylase [18].

HIF activity is also regulated in an oxygen-dependent manner by an asparaginyl hydroxylase, Factor Inhibiting HIF-1 (FIH1), which hydroxylates an asparagine in the C-terminal activation domain (CAD) of the \( \alpha \) subunit (N-803 in human HIF-1\( \alpha \)) under normoxia and mild hypoxia. Hydroxylation of N-803 blocks interactions between HIF-1\( \alpha \) and p300/CBP coactivators preventing HIF activation [19]. Because FIH1 is less sensitive to oxygen drop than PHDs, it remains active under moderate hypoxia and keeps inactive the HIF-\( \alpha \) molecules that avoid the PHD-mediated degradation that occurs in mild hypoxia [20].

PHDs and FIH1 are dioxygenases that, in addition to molecular oxygen, require \( \text{Fe}^{2+} \) and 2-oxoglutarate (2-OG) co-factors for activity [20]. This provides an opportunity for regulation.
of their activity and in turn HIF-1α levels/HIF activity. Iron chelation is frequently used for activation of HIF-1 in cell culture [20]. There has also been some evidence of mitochondrial involvement in HIF regulation in that, under moderately hypoxic conditions, mitochondria will produce reactive oxygen species (ROS) from complex III of the electron-transport chain, which inhibit the activity of PHDs by oxidizing Fe²⁺ to Fe³⁺, thus accelerating the stabilization of HIF [21,22]. 2-OG is an intermediate in the tricarboxylic acid (TCA) cycle and structurally related compounds (e.g., dimethyloxalylglycine or other TCA cycle intermediates, fumarate and succinate) can inhibit activity of PHDs directly by reversibly competing for the active site with 2-OG or indirectly via increasing cellular levels of ROS [23]. Recently, heterozygous mutations in IDH1 or IDH2 genes were described in glioma and acute myeloid leukemia. Mutant proteins, acting in a dominant-negative fashion, are defective in their ability to oxidize isocitrate to 2-OG. An initial report suggested that in cells with mutant IDH HIF-1α is indirectly stabilized because reduced synthesis of 2-OG lessens PHD function [24]. However, further studies found comparable levels of 2-OG in both wild-type and mutant cells; instead, the mutant IDHs acquire a neomorphic activity, which reduces 2-OG to 2-hydroxyglutarate (2-HG) [25]. Moreover, it was reported that (R)-2-HG, the product of mutant IDH, can be utilized in place of 2-OG in the active center of PHDs. Here, (R)-2-HG (but not (S)-2-HG which acts as an inhibitor of PHDs) is oxidized into 2-OG, resulting in increased PHD activity and correspondingly decreased HIF-α levels and HIF activity [26]. More work will be required to finalize the functional consequences of IDH mutations on activity of the HIF pathway and tumor growth.

Finally, HIF-α is situated at the convergence of multiple oncogenic and tumor suppressor pathways, including the PI3K/AKT and MAPK/ERK pathways, which regulate HIF nonspecifically in an oxygen-independent manner. Activation of the PI3K/AKT pathway has been demonstrated to increase translation of HIF-1α mRNA and HIF-1α production [27,28]. Tumor suppressors (p53, GSK3β, and so forth) interfere with HIF function by decreasing HIF-1α stability or transcriptional activity [23]. One important type of regulation is that of HIF-1α phosphorylation, which can affect both HIF-1α stability and its transactivation potential. HIF-1α can be phosphorylated by GSK3 at three serine residues (551, 555 and 589) within the human HIF-1α N-terminal transactivation domain [29,30]. This recruits Fbw7 and USP28, which then mediates HIF-1α ubiquitination and subsequent VHL-independent proteasomal degradation [31]. PLK3 also destabilizes HIF-1α by phosphorylation at two serine residues (576 and 657) [32]. Conversely, phosphorylation of HIF-1α can have a stabilization effect. It has been demonstrated that ATM can phosphorylate HIF-1α at Ser-696, which increases its stability [33]. HIF-1α is also phosphorylated in its CAD by ERK1, which increases its transcriptional activity, but not its stability [34]. Thr-796 in HIF-1α is phosphorylated by CK2 and this phosphorylation is also important for its transactivation potential and not stability [35,36]. The p42 and p44 MAPK pathways regulate HIF-1α post-translationally by phosphorylating two serine residues (641 and 643). This phosphorylation promotes nuclear accumulation of HIF-1α, which leads to an increase of HIF-1-activated transcription [37,38]. In addition, HIF-1α Ser 247 phosphorylation coregulates the dimerization of HIF-1α/HIF-1β complex. HIF-1α can be phosphorylated on Ser-247 in the PAS-B domain by CK1, which destabilizes the HIF-1α/-1β complex, and thus diminishes its transcriptional activity [39]. HIF-1α/-1β dimerization is also regulated by means other than HIF-1α phosphorylation. For example, COMMD1 binds to the N-terminal domain of HIF-1α, competing with HIF-1β binding, and subsequently decreases the DNA binding and transcriptional activation of the complex [40]. Finally, the HIF-1α/p300 interaction is also post-translationally regulated. Both hydroxylation of asparagine 803 and S-nitrosylation of cysteine 800 in the C-TAD of HIF-1α decrease p300 binding, while phosphorylation of Thr-796 in the C-TAD does not affect p300 binding [41].
Distinct roles of HIF-α subunits

Among the three members of the HIF-α family, HIF-1α and HIF-2α (also called EPAS1, MOP2 or HLF) have been extensively studied, whereas significantly less is known about the third member, HIF-3α. HIF-α proteins exhibit high conservation in overall amino acid sequence, as well as similar domain structure, mechanisms of activation, heterodimerization with HIF-1β, and binding to the same HIF-binding site [20]. HIF-1α and HIF-2α each contain an N-terminal activation domain and CAD, and both act as positive transcriptional regulators. In contrast, the truncated HIF-3α lacks a CAD and acts as a dominant negative regulator of transcription and its expression is suppressed in renal cell carcinoma [23].

Despite the above-mentioned similarities, there is mounting evidence that HIF-1α and HIF-2α subunits are functionally distinct. The fact that neither HIF-1α−/− nor HIF-2α−/− embryos can survive suggests a lack of functional complementation between the two isoforms [42]. The ubiquitous expression of HIF-1α, as opposed to the more cell type-specific expression of HIF-2α, also suggests different physiological roles. Knockdown of HIF-1α or HIF-2α by siRNA elicits remarkably different cell-specific effects: in endothelial and breast cancer cells hypoxia-inducible gene expression has been demonstrated to be critically dependent on HIF-1α but not HIF-2α [43]; whereas in renal carcinoma cells, expression was critically dependent on HIF-2α [44]. HIF-α isoforms display unexpected reciprocal suppressive interactions in renal cell carcinoma: enhanced expression of HIF-2α suppresses HIF-1α and vice versa [44].

In certain cancers, HIF-1α and HIF-2α have been shown to play contrasting roles in tumorigenesis. Contrary to earlier conclusions, HIF-1α expression was found to correlate with lower disease stages in clear cell renal cell carcinoma (ccRCC), non-small-cell lung cancer, head and neck squamous cell carcinoma and neuroblastoma; whereas HIF-2α expression was found to correlate with more advanced stages and was consistently scored as a negative prognostic factor [45]. The most compelling evidence about the distinct roles of HIF-1α and HIF-2α has been accumulated in the VHL-deficient ccRCC: while ccRCC always overexpress HIF-2α and sometimes overexpress HIF-1α, only HIF-2α is required for their growth [46]. In addition, both HIF-1α and HIF-2α, stabilized upon VHL inactivation, can transcriptionally down-regulate transcription of HIF-1α in ccRCC through a mechanism that involves binding of either subunit to a reverse HRE in the HIF-1α proximal promoter followed by a series of repressive histone modifications [47]. This observation is in agreement with previous reports suggesting that in the early stages of ccRCC HIF-1α provides a metabolic advantage due to activating glycolytic enzymes, but hinders progression by inducing cell-cycle arrest or apoptosis. Inactivation of HIF-1α and its replacement with HIF-2α discontinues this inhibitory effect and ccRCC rapidly progresses [45,48,49]. Together, these data support the notion that HIF-2α has a greater oncogenic potential than HIF-1α in ccRCC [50].

Distinct functions of HIF-1α & -2α in certain types of cancer can be explained by differential direct & indirect transcriptional effects

HIF-1α and HIF-2α activate different sets of transcriptional targets (direct transcriptional effect). Several studies have shown that HIF-1α and HIF-2α differ in their capability to transactivate hypoxia-inducible genes. Some genes were transactivated exclusively by HIF-1α (notably glycolytic and proapoptotic genes) whereas others were transactivated by both isoforms [43,51]. HIF-2α, on the other hand, is a more efficient activator of genes encoding stem cell markers (see below). In a report that used siRNA methodology, HIF-2α preferentially regulated a small group of genes that had in common binding sites for the ETS family of transcription factors in their promoter regions. Knockdown of ELK-1, the most prominent member of the ETS family, significantly reduced hypoxic induction of the
HIF-2α-dependent genes [52]. A number of regulatory elements (e.g., ATF-1, CREB-1, AP-1 and Sp1) have been described to cooperate with HREs, [16]; however data on whether their cognate factors preferentially engage in cooperation with HIF-1α or HIF-2α are scarce. Together, these studies suggest that, of the two isoforms, HIF-1α is a more universal activator [51], whereas HIF-2α is the more selective activator of transcription and the observed significant quantitative differences/specificity of activation could be accounted for by preferential cooperation of one isoform with transcription factors binding to the regulatory elements juxtaposed to HREs [23]. Sirtuins, a family of stress-responsive nicotinamide adenine dinucleotide-dependent histone deacetylases that serve as sensors of the cellular redox state, also control transcriptional activity of HIF-1α and HIF-2α [23]. Sirt1 was reported to deacetylate both HIF-1α and HIF-2α with different functional outcomes: deacetylation of HIF-1α decreases its transcriptional activity [53], whereas deacetylation of HIF-2α resulted in enhanced transcriptional activity [54]. Thus, conditions of cellular redox/metabolic stress fine-tune activity of HIF-1α and HIF-2α.

HIF-1α and HIF-2α differentially modulate the activity of other transcription factors (indirect transcriptional effects). HIF-1α and HIF-2α have been reported to modulate (through direct or indirect interactions) the transcriptional activity of certain critical oncogenes/tumor suppressors with opposite outcomes. Notable in this respect is MYC, the function of which is antagonized by HIF-1α but enhanced by HIF-2α [46,55]. Differential regulation of the tumor suppressor protein p53 by HIF-1α and HIF-2α was also reported. On the one side, HIF-1α directly binds p53, inducing its stabilization, and eventually causing cell death [56,57]. Conversely, inhibition of HIF-2α has been shown to promote p53 activity and higher levels of HIF-2α contribute to increased radio- and chemoresistance [58]. In this way, HIF-1α and HIF-2α can achieve opposing effects on tumor behavior through indirect transcriptional effects.

HIF-1α and HIF-2α also have different effects on CSCs, also known as tumor-initiating or tumor-propagating cells, which are undifferentiated cells with the capacity to self-renew and reconstitute tumors in vivo that are phenotypically similar to the parental tumor [59]. An important feature of CSCs is their enhanced resistance to radio- and chemotherapy that limits DNA damage, allowing them to survive the treatment and repopulate the tumor with their progeny. CSCs thus represent an important therapeutic target and their complete elimination is expected to greatly enhance the treatment efficacy [59,60]. There are numerous reports implicating hypoxia and HIFs in CSC biology. For example, populations of cells enriched for CSCs can be isolated from glioblastoma tumors using the cell surface marker CD133 and hypoxia may promote the expansion of the CD133-positive cells through activation of HIF-1 [61]. Other reports highlight the prominent role of HIF-2α in CSCs: this isoform is selectively expressed in CSCs, whereas HIF-1α was expressed at comparable levels in both CSC and non-CSC (CD133-negative) populations [62]. In the same study, only targeting HIF-2α by shRNA decreased the growth of CSC populations in vitro, inhibited CSC-mediated angiogenesis and significantly prolonged the survival of mice intracranially implanted with CSCs. In a separate study, HIF-2α colocalized with neural crest markers in a subset of immature cells in neuroblastoma tumors and HIF-2α knockdown induced differentiation of these cells [63]. HIFs thus help maintain an undifferentiated state in some populations of CSCs as well as other stem and progenitor cells [60]. HIFs apparently control self-renewal and differentiation processes in these cells by means of trans-activating critical genes and the key transcription factors involved in these processes. In this category, interaction of HIF-1α with Notch1 helps maintain the undifferentiated cell state [64], whereas HIF-2α induces expression and transcriptional activity of the marker of the undifferentiated state – Oct4 [65]. HIF-2α also regulates the transcription factor Sox2 [66], which, in turn, controls pluripotency by direct regulation of Oct4 levels [67]. Recently, reprogramming of somatic cells into induced pluripotent cells was achieved through
transduction of four defined transcription factors (c-MYC, KLF4, SOX2 and Oct4) all of which are transactivated by HIF [55,65,66]. Together, these reports provide evidence that HIFs directly control markers of the undifferentiated state (stemness) and are necessary for maintaining CSCs. Due to its critical role and selective activation, HIF-2α could be an important therapeutic target in CSCs.

Genes activated by HIF

Along with its co-activators, the HIF complex acts as a transcription factor for hundreds of genes, including VEGF, NOS, GLUT1, LDH, CA9 and MDR1 (Table 1) [69-107]. Many of these genes affect cancer progression through angiogenesis, erythropoiesis, increased glucose metabolism, immune evasion, immortalization, genetic instability, increased invasion and metastasis, pH regulation, drug resistance, CSC maintenance and proliferation.

Two of the most essential functions of cancer biology that HIF-1 activates are angiogenesis and glucose metabolism [108]; the former controls oxygen and nutrient delivery and the latter generates energy and synthetic intermediates for growth and survival. VEGF is an important growth factor in angiogenesis and vascularization and plays a significant role in hypoxic conditions and has been demonstrated to increase in vivo tumor size and vascularization [109]. VEGF also plays a critical role in embryonic development – deletion of a single allele of the gene in VEGF-knockout mice is lethal within two weeks of development. HIF-1 activates VEGF transcription by binding to its HRE and VEGF synthesis increases angiogenesis [110]. NOS produces nitric oxide, which promotes angiogenesis and cell proliferation, and increases cell survival by inhibiting apoptosis. HIF-1 binds to the NOS HRE and thus upregulates NOS expression and increases cancer progression [111]. Histological studies have associated increased malignancy and aggressiveness in cancers with increased HIF-1 and VEGF expression, and NOS expression has been confirmed in many cancers, including breast, head and neck, prostate and colorectal [111,112].

HIF-1 is a transcription factor for many genes involved in the glycolytic cascade, including GLUT1 and LDH, which allow tumors to grow under hypoxic conditions by metabolizing glucose to lactate through anaerobic glycolysis [92,113]. One result of anaerobic glycolysis is decreased production of ROS generated by oxidative phosphorylation, which can prevent cellular senescence, and thus remove a constraint on tumor growth [114]. Another consequence of glycolysis and lactate buildup is hypoxic acidosis, which, if not prevented, would lead to a decrease in intracellular pH, cell damage and death. However, HIF-1 activates the synthesis of monocarboxylate transporters (e.g., MCT) that extrude lactate into the extracellular space [115] and CA-9 and CA-12, which use CO2 to generate HCO3− to dampen the effects of acidosis and increase cell survival. Not surprisingly, higher GLUT1 levels are correlated with poor survival in many cancers – including breast, head and neck, esophageal, stomach, bladder, ovarian, colorectal, and non-small-cell lung carcinomas – and CA-9 is used as a marker for cancer progression [1,116].

In addition to activating gene expression for cancer progression, HIF-1 decreases the effectiveness of anticancer therapies such as radiation and chemotherapy. HIF-1 itself is stabilized by radiation, even doubling its activity in the two days following therapy, due to the re-oxygenation and release of ROS that follows radiation treatment [117,118]. The radiation-induced production of ROS increases HIF-1α accumulation by reducing its degradation through an AMP-activated protein kinase (AMPK)-dependent pathway, where ROS-activated AMPK inhibits the interaction between HIF-1α and VHL, thus preventing ubiquitination and subsequent degradation [119,120]. Increased HIF-1α levels have also been shown to decrease the effectiveness of chemotherapy in various types of cancer [121-124], due to factors such as HIF-mediated regulation of drug efflux, cell proliferation.
and survival, metabolic reprogramming and inhibition of DNA damage [125]. One specific mechanism by which HIF-1 can decrease effectiveness of therapy is by activation of MDR1 and the expression of the multiple drug resistance phenotype, which is present in the most aggressive cancers and correlates with the metastatic ability of multiple solid tumor cancers [126]. In addition, many anticancer therapeutics, such as doxorubicin, rely on the generation of ROS for cytotoxicity, but ROS stabilize HIF-1α [127]. Moreover, many therapeutics contain weakly basic moieties that can react with the acidic tumor microenvironment and lose efficacy [128].

Molecular targets of HIF-1 inhibitors

There are many different strategies for inhibiting the HIF-1 pathway. Small molecules have been shown to inhibit HIF activity through a variety of mechanisms including HIF-1α protein synthesis, HIF-1α protein stabilization, HIF-1α–HIF-1β dimerization, HIF-1 dimer DNA binding, and interactions with other proteins. There were two comprehensive reviews in 2012 by Semenza [129] and Xia et al. [130] that cover these mechanisms in detail and give fairly exhaustive lists of the small molecules that inhibit at each level.

Many HIF-1 inhibitors affect the HIF-1 pathway upstream of HIF-1α synthesis (Figure 2). Cancer metabolism and regulation is a very complex system and it can be difficult to separate the desired effects on one pathway from another. This complexity can work for medicinal chemists in a synergistic manner by up- or down-regulating multiple pathways/targets by producing similar net results in the design and implementation of therapeutics. Conversely, the inhibitors may have unexpected counter-productive effects which may contribute to the high late-stage clinical failures for anticancer drugs, which is around 70% for Phase II trials and 60% for Phase III trials [131]. These high attrition rates can, moreover, be attributed to several factors, including the hypoxic and acidic tumor microenvironment; veracity and fidelity of in vitro preclinical models; drug absorption, distribution, metabolism, excretion, and toxicity; drug delivery in vivo; and translation to the clinic [132].

In the field of HIF-1 inhibitors, the role of hypoxia is key. Tumor hypoxia has been touted as “the best validated target that has yet to be exploited in oncology” [5]. The definition of hypoxia has been well established at 0–5% oxygen, but there is a variance in the levels of oxygen defining normoxia. Atmospheric oxygen levels are approximately 20%, whereas tissues in the body have normal oxygen levels that range from 0 (bone marrow) to 14% (lung, liver, kidney and heart), with levels of approximately 10–12% in circulation [133]. Thus, in vitro assays that are carried out under ‘normoxic’ conditions, but are simply left open to the atmosphere ought to be considered ‘hypoxic’ when compared to in vivo oxygen levels [134]. This difference in oxygen levels can contribute negatively to many preclinical studies and should be considered as a contributing factor to drug attrition.

Another factor in tumor hypoxia is the acidic extracellular environment due to the increased expression of the glycolytic phenotype. This creates a unique pH gradient for tumors that is the opposite of that of normal tissue [135]. It has been demonstrated that weakly basic anticancer therapeutics, such as doxorubicin, are excluded from acidic tumors [136]. This acidic micro environment can also contribute negatively to in vivo studies and should be considered as another factor contributing to drug failure.

HIF-1 inhibitors in the clinic: hits & misses

Transcription factors have, for a long time, been considered undruggable targets, and to date, no specific inhibitor of HIF has been brought to market. Multiple levels of regulation and the fact that multiple signaling pathways converge on HIF-α explain the diversity of
compounds that, among other targets, inhibit HIF. Although there are many challenges to targeting HIF, there have been a number of compounds that have made it to clinical trials as anticancer therapeutics, which have also been shown to inhibit HIF activity. Some of these have failed their trials, while others have been US FDA approved for patient treatment. The following illustrates a few of these examples (Table 2) and what their outcomes say for the field as a whole.

Camptothecins: camptothecin, topotecan, irinotecan

In the 1960s, camptothecin was first isolated from the bark of a native Chinese tree, *Camptotheca acuminata*, by Wall and Wani. By the mid 1970s, camptothecin was in clinical trials due to its anticancer activity, but it was terminated because of its serious side effects [137]. Research on camptothecin was not resumed until the discovery of DNA topoisomerase 1 (TOP1) as its primary target and the successful development of water-soluble derivatives of camptothecin: topotecan (Hycamtin®) and irinotecan (Camptosar, CPT-11) [138]. The camptothecins have a unique mechanism of action that targets TOP1, an essential human enzyme that relaxes DNA supercoiling by forming single strand breaks, unwinding, then religating the strands back together. The camptothecins act as irreversible inhibitors to the TOP1–DNA complex by intercalating into DNA at the protein–DNA interface, which then prevents the religation of the DNA strands [139]. Both irinotecan and topotecan have similar mechanisms of action as camptothecin. TOP1 is an upstream regulator of the HIF-1 pathway. TOP1 inhibition leads to HIF-1α down-regulation due to decreased protein accumulation and translation that is independent of oxygen or proteasomal degradation [140]. It has been suggested that this mechanism could be due to the activation of a novel antisense transcript from the 5′ end of *HIF-1α* and the removal of Pol II from the promoter-proximal pause site of the *HIF-1α* gene [141]. The mechanism of inhibition of HIF-1α translation by camptothecin and its derivatives is TOP1-dependent but DNA-damage independent. Irinotecan was approved for treatment of colorectal cancer in 1996 [142] and topotecan was approved for the treatment of Stage IVB recurrent carcinoma of the cervix in 2006 [219]. In 2007, the FDA approved Hycamtin for treatment of relapsed small cell lung cancer [304]. The successful development of these camptothecin derivatives would not have been possible without further elucidation of their mechanism of action. This case illustrates how drugs (and classes of drugs) that have unknown mechanisms of action and have previously failed clinical trials can be repurposed and brought to market once the mode of action is elucidated and the pharmacokinetics optimized.

Bortezomib

Bortezomib (Velcade®, PS-341) is a dipeptide boronic acid-containing compound that reversibly inhibits the chymotryptic activity of the 20S subunit of the 26S proteasome due to the high binding affinity and specificity between the boron atom and the 20S subunit [143]. The proteasome regulates protein expression and function by degradation of ubiquitinated proteins, and cleanses the cell of unfolded or misfolded proteins. Bortezomib has been shown to directly inhibit proliferation and induce apoptosis in multiple myeloma cell lines and patient tumor cells resistant to conventional therapies [144]. Proteasome inhibition by bortezomib leads to disruption of intracellular protein metabolism. The downstream biological effects of proteasome inhibition are numerous, with direct effects on both multiple myeloma cells and their micro environment, including inhibition of cytokine secretion, suppression of adhesion molecule expression and inhibition of angiogenesis [145]. Because bortezomib’s primary target is the proteasome, it prevents the degradation of ubiquitinated HIFα and leads to the accumulation of HIF-1α under normoxia. However, the stabilized HIF-1(−2) is inactive due to upregulation of FIH activity, which inhibits recruitment of p300 [146]. There has been other evidence that bortezomib does not directly affect the formation of the HIF-1–p300 complex, but interferes with the C-terminal domain.
of HIF-1α [147]. More recently, bortezomib was shown to inhibit the PI3K/AKT/mTOR pathway upstream of the HIF pathway in prostate cancer cells [148]. Due to bortezomib’s remarkable clinical activity against multiple myeloma, it was rapidly approved by the FDA in 2003 to treat relapsed and refractory multiple myeloma [149]. It is unknown whether HIF inhibition is critical to the clinical activity of bortezomib. Bortezomib is an excellent example of an FDA-approved anticancer therapeutic that indirectly inhibits HIF expression and function and that has differing modes of action in different cancer models, which illustrates the complexity of the field.

**Romidepsin**

Romidepsin (Istodax®, FK228, FR901228) is an anticancer agent isolated from the bacterium *Chromobacterium violaceum* that was first reported by Fujisawa Pharmaceutical Company (now Astellas Pharma) in 1994 [65]. Its mechanism of action was demonstrated in 1998 [150,151]. As a prodrug with a disulfide bond, romidepsin undergoes reduction to release a zinc-binding thiol [152]. This thiol reversibly interacts with a zinc atom in the binding pocket of Zn-dependent histone deacetylases (HDACs) to block their activity, classifying romidepsin as a HDAC inhibitor (HDACI). HDACs affect gene expression by removal of acetyl groups from acetylated lysine residues in histones. HDACs also deacetylate nonhistone proteins, such as transcription factors. *In vitro*, romidepsin causes the accumulation of acetylated histones, thus inducing cell cycle arrest and apoptosis of some cancer cell lines with IC_{50} values in the nano molar range [153]. HDAC activity is crucial for the transactivation potential of HIF-1α and most HDACIs regulate acetylation of the HIF-1α/p300 complex [154]. It has also been suggested that the class II isozyme HDACs are involved in direct acetylation and ubiquitination of HIF-1α [155]. Romidepsin was approved as a treatment for cutaneous T-cell lymphoma in 2009 [305]. Other HDACIs are also in preclinical and clinical development, but many in the field have moved to target other mechanisms of action due to high adverse effects associated with the HDACIs.

**Temsirolimus**

Temsirolimus (Torisel®, CCI-779), the ester version of rapamycin, is the first FDA approved inhibitor of mammalian target of rapamycin (mTOR/TORC1), which is a serine/threonine-specific kinase in the phosphatidylinositol 3–kinase (PI3K) related protein family [156]. In RCC, temsirolimus was shown to inhibit tumor growth and HIF expression by inhibition of the mTOR-dependent kinase cascade needed for HIF mRNA translation [157]. Inhibition of PI3K and its downstream target mTOR was demonstrated in prostate cancer (PC-3) cells to decrease HIF-1-dependent gene expression and temsirolimus was shown to suppresses HIF-1 activation by increasing HIF-1α degradation rate in hypoxic PC-3 cells [158]. The FDA approved Torisel (temsirolimus) for the treatment of RCC in 2007 [306]. However, not all inhibitors of the PI3K/AKT/mTOR pathway have had such success.

**Perifosine**

Perifosine is an alkylphospholipid analogue, which has demonstrated significant antiproliferative activity in several human tumor model systems both *in vitro* and *in vivo*. It has a similar structure to miltefosine, a drug that has been approved in Europe for the treatment of cutaneous lymphomas and metastasis from breast cancer. Perifosine’s activity is also due to its effect on the PI3K/AKT/mTOR pathway, an upstream regulator of the HIF pathway. Many growth factors that upregulate HIF activate the PI3K–AKT–mTOR pathway, which leads to increased HIF-1α protein translation and stability [159]. Perifosine, which inhibits AKT in a dose-dependent manner, entered Phase I clinical trials in 2003 [160] and, in 2010, reached Phase II trial due to significant growth inhibition *in vitro* and *in vivo* in the Waldenstrom macro globulinemia model [161]. In 2012, however, perifosine...
failed a late-stage Phase III study on colorectal cancer due to lack of efficacy but will continue in another Phase III study as part of combination therapy with bortezomib against multiple myeloma [307]. Perifosine is one of many compounds indirectly affecting HIF expression that showed great promise in preclinical models, but has failed to exhibit efficacy in human trials.

2-methoxyestradiol

2-methoxyestradiol (2ME2) is a natural metabolite of estradiol and is a potent antitumor agent, due to its antiproliferative, antimetastatic and antiangiogenic activity. These antitumor activities result from its pro-apoptotic activity, microtubule activity and production of superoxides [162]. Its main potency appears to be derived from disruption of cellular microtubules that are necessary for HIF-1α translocation to the nucleus. 2ME2 thus retards HIF-1 nuclear accumulation and activity in a manner that is both oxygen- and proteasome-independent [163]. It was subsequently found that microtubule inhibitors such as 2ME2 and taxol also inhibit HIF-1α mRNA translation [164]. A Phase I clinical trial of 2ME2 was successfully concluded in 2006 [165]. In 2011, 2ME2 nanocrystal dispersion failed to show a significant effect in a Phase II study against castration-resistant prostate cancer [166] and in 2012, in a second Phase II study against metastatic renal cell carcinoma due to both lack of objective effects and high toxicity. The clinicians recommended halting trials of 2ME2 in favor of a new 2ME2 analog in development [167]. Hopefully, this new analog, ENMD-1198, will have more favorable results.

Echinomycin

Echinomycin (quinomycin A, NSC 526417) is a cyclic peptide antibiotic agent of quinoxaline that was originally isolated from Streptomyces echinatus [168]. Echinomycin is known to bind DNA in a sequence-specific manner. Binding sites for echinomycin contain the central sequence 5′-CG-3′ and the key recognition elements are contained in the sequences 5′-ACGT-3′ and 5′-TCGT-3′, which are part of the DNA recognition motifs for several transcription factors [169]. Inhibition of HIF-1 binding to the HRE (RCGTG; where R = purine A or G), a step required for induction of transcription, is a potential mechanism by which echinomycin may inhibit HIF-1 activity [170]. Echinomycin was shown in chromatin immunoprecipitation experiments to selectively inhibit binding of HIF-1 to DNA [171]. Despite echinomycin’s in vitro apoptosis-inducing activity and the initial report of its in vivo antitumor effect in mice, echinomycin’s clinical development was halted in the late 1980s following extensive testing as a cytotoxic agent in Phase I-II trials, which failed to show significant activity [172].

Ansamycins: geldanamycin, 17-AAG, 17-DMAG

Geldanamycin (GA) is a macrocyclicpolyketide antibiotic containing a benzoquinonone moiety and was originally isolated from Streptomyces hygroscopicus [173]. GA is a Hsp90 inhibitor, that acts by binding the N-terminal ATP-binding domain of Hsp90, leading to the destabilization and eventual degradation of Hsp90 client proteins [174-176]. Hsp90, one of the most abundant cellular proteins, assists in protein folding and degradation and is upregulated during cellular stress [177,178]. HIF is a client protein of Hsp90 and the inhibition of Hsp90 has been shown to destabilize HIF, leading to HIF degradation and decrease in transcriptional activity [179,180]. GA and its derivatives 17-AAG (tanespimycin) and 17-DMAG (alvespimycin) have demonstrated anti-tumorigenic and antiangiogenic properties both in vitro and in vivo. However, GA was never brought to the clinic due to its poor pharmacological properties and hepatotoxicity in animal models [181,182]. 17-AAG was the first-in-class Hsp90 inhibitor to enter Phase I trials, where it showed promise; however, it showed poor results in Phase II trials, most likely due to its poor bioavailability.
and solubility [183-186]. 17-DMAG, an orally available agent, has shown promise in the clinic, with success in Phase I trials, but needs further evaluation [187,188].

**Conclusion**

Hypoxic conditions in the cancer microenvironment lead to increased resistance to both chemotherapy and radiotherapy. In most cases, the HIF pathway is the primary pathway responsible for this more malignant phenotype and its activation leads to increased cancer metastasis and poor patient prognosis. There has been much effort in this area to develop small-molecule inhibitors of the HIF pathway (mostly focused on HIF-1) as well as upstream and downstream effects. There have been no approved drugs that directly inhibit the HIF pathway, but there have been a few that indirectly affect the HIF pathway, as well as many more that have failed to demonstrate therapeutic efficacy in clinical trials for cancer patients. Some of these failures can be accredited to a lack of specificity and/or redundancy in the complexity of tumor signaling/metabolism that can overcome the inhibition effects. Another contributing factor to the failure of these compounds can be attributed to the lack of patient selection in clinical trials. Although many clinical trials evaluate the efficacy of anticancer therapeutics and examine their effects on HIF levels, patients are not selected based on their HIF-expression levels. If patients do not have elevated levels of HIF, therapeutics that target the HIF pathway may be less effective. More work needs to be done to identify novel, potent and more specific inhibitors targeting clearly defined points in the HIF pathway. Such new agents should be used in combination therapy and will hopefully overcome resistance that may develop during the initial treatment.

**Future perspective**

Recently, the HIF pathway was touted as “technically undruggable or at the very least as extremely challenging to target by medicinal chemists using small molecules” [189]. This attitude can be attributed not only to the difficulty of targeting such a complex pathway, but also to the challenges of targeting protein–protein interactions [190]. Small molecules typically inhibit protein function by binding with high affinity and specificity to hydrophobic pockets on or near the protein’s surface. When trying to disrupt interactions between proteins, these binding sites may no longer be accessible and the interactions between proteins are so multi-farious that one small molecule may not be able to interrupt the key interactions. There have been advances in this field of targeting protein–protein interaction, such as stapled peptide inhibitors of transcription factors [191] and small-molecule inhibitors of the MDM2-p53 interaction as anticancer therapeutics [192]. Some interesting features of the small molecules identified are that they tend to be large, lipophilic, and rigid structures with complex 3D shape that form few hydrogen bonds [193,194]. These structural differences vary from the typical Lipinski rule of five for drug-like properties [195] and could portend a new paradigm for drug-like qualities for small molecules that inhibit protein–protein interactions.

Interest in small-molecule inhibitors of the HIF pathway has steadily increased over the past 15 years. The number of patent applications, publications, citations and clinical trials has risen since the late 1990s and early 2000s (Figure 3). Although there have been few drugs that have made it all the way through clinical trials, the field is ripe and interest is increasing. It still remains to be seen whether inhibitors capable of distinguishing HIF-1 and HIF-2 complexes can be developed. In addition, new therapeutics are being developed against downstream HIF targets, such as MCT (91) and CA-IX (196). Inhibitors of post-translational modifications of HIF-1α have also shown some promise. Kaempferol was recently shown to inactivate the p44/42 MAPK phosphorylation of HIF-1α, causing its mislocalization into the cytoplasm in hepatocellular carcinoma [197]. Results from high-
throughput screenings and natural product discovery [198] have also yielded new hits and lead compounds, but even more new methods need to be identified. Recently, novel HIF inhibitors from frankincense were identified through a new method using a molecularly imprinted polymer [199]. Acriflavine was identified as a HIF-dimerization inhibitor from a screening of drugs that had previously made it to Phase II clinical trials [200]. In addition, targeting the HIF-2α/-1β dimerization has been suggested as a valid target for anticancer therapeutics [201]. The well-known HIF/p300 interaction inhibitor chetomin [202] and its family, the epidithiodiketopiperazines, act via a mechanism of action involving disruption of the zinc-binding sites in the CH1 domain of p300 [203]. In addition, artificial α-helices have been demonstrated to interfere with the HIF/p300 interaction [204]. Oncolytic viruses dependent upon HIF expression for their replication have also been developed and showed strong antitumor effects [205-207]. Recently, a group of collaborators, including the authors, reported a new class of HIF-1 inhibitors, that appears to target the interaction between HIF-1 and p300 and, therefore, HIF-mediated transcription [208-214]. The lead arylsulfonamide compound (KCN1) showed potent anticancer activity in several cancer models [215,216]. Moreover, these inhibitors do not show intrinsic cytotoxicity and thus are promising compounds for further clinical development.

**Key Terms**

**Cancer stem-like cells**
Undifferentiated cells with the capacity to self-renew and reconstitute tumors in vivo that are phenotypically similar to the parental tumor.

**Hypoxia-inducible factor**
Transcription factors regulated by the presence or absence of oxygen.

**Hypoxia**
Reduction of oxygen supply to tissues.

**Angiogenesis**
Process of new blood vessel formation from existing vasculature.

**Drug-like**
Following Lipinski’s rule of five, no more than five hydrogen bond donors, no more than ten hydrogen bond acceptors, molecular weight of less than 500, logP of less than 5.

**References**

Papers of special note have been highlighted as:

■ of considerable interest

study to show that hypoxia selects for tumor cells with mutations in the p53 gene that prevent hypoxia-induced apoptosis.


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203. Cook KM, Hilton ST, Mecinović J, Motherwell WB, Figg WD, Schofield CJ. Epidithiodiketopiperazines block the interaction between hypoxia-inducible factor-1α (HIF-1α) and p300 by a zinc ejection mechanism. J. Biol. Chem. 2009; 284(39):26831–26838. [PubMed: 19589782]


Websites


Executive summary

Hypoxia in cancer

- Hypoxia is an important element in the tumor microenvironment.
- Hypoxia leads to a more malignant phenotype that is: resistant to radiation and chemotherapy; more invasive; genetically unstable; resistant to apoptosis; greater metastatic potential.

The hypoxia inducible factor pathway

- The hypoxia inducible factor (HIF) pathway is a critical target for anticancer therapeutics.
  - The HIF complex acts as a transcription factor for many genes that increase tumor survival, including pro-angiogenic and pro-glycolytic genes.
  - Increased HIF-1α levels in tumors lead to decreased patient survival rate.
- Distinct roles of HIF-α subunits.
  - HIF-1α, HIF-2α, and HIF-3α all have differing roles and effects on cancer progression.
- Distinct functions of HIF-1α and HIF-2α in certain types of cancer can be explained by differential direct and indirect transcriptional effects.
  - HIF-1 and HIF-2 activate different sets of transcriptional targets; HIF3 is an inhibitor.
  - >100 different genes have been identified as HIF targets, with a variety of functions such as angiogenesis, erythropoiesis, increased glucose metabolism, immune evasion, immortalization, genetic instability, increased invasion and metastasis, pH regulation, cancer stem cell maintenance, and proliferation.

Molecular targets of HIF-1 inhibitors

- HIF inhibitors target the HIF pathway by a variety of mostly indirect mechanisms: HIF-1α protein synthesis, HIF-1α protein stabilization, HIF-1α–HIF-1β dimerization, HIF-1 dimer DNA binding and interactions with other proteins.

HIF-1 inhibitors in the clinic: hits & misses

- Many anticancer agents with indirect HIF-1 inhibition have made it to clinical trials and a few have been US FDA-approved for cancer treatment, such as: camptothecins: camptothecin, topotecan, irinotecan; bortezomib, romidepsin, temsirolimus, perifosine, 2-Methoxyestradiol, echinomycin; ansamycins: Geldanamycin, 17-AAG, 17-DMAG.

Conclusion

- More work needs to be done to identify novel, potent and more specific inhibitors targeting clearly defined points in the HIF pathway.

Future perspective
| ■ | Interest in the HIF field is increasing and looking to new mechanisms for inhibition will be key to this field. |
Figure 1. The hypoxia inducible factor-1
Under normoxic conditions, HIF-1α is rapidly hydroxylated by prolyl hydroxylases, which mediates binding by the VHL ubiquitin ligase complex, addition of poly-Ub which tags it for proteosomal degradation. Under hypoxic conditions, prolyl hydroxylases cannot hydroxylate HIF-1α, preventing VHL binding which leads to HIF-1α stabilization. HIF-1α then heterodimerizes with HIF-1β, recruits the p300/CBP co-factors and forms an active HIF transcription complex in the nucleus that binds to HREs on target genes and activates their transcription.

Figure 2. Mechanisms of hypoxia inducible factor inhibition

Figure 3. Trends of hypoxia inducible factor inhibitors from 2000–2012
(A) Number of patents issued by year. (B) Number of publications by year. (C) Number of clinical trials by year. (D) Number of citations by year.
Data taken from [308-310].
Table 1
Selected hypoxia inducible factor target genes and their effects on cancer progression

<table>
<thead>
<tr>
<th>Effect on cancer progression</th>
<th>Genes activated by hypoxia inducible factor-1</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Angiogenesis</td>
<td>ANGPT2, C-MET, ID2, LEP, NOS, PDGF, SCF, SDF-1, VEGF</td>
<td>[74-82]</td>
</tr>
<tr>
<td>Drug resistance</td>
<td>MDR1</td>
<td>[83]</td>
</tr>
<tr>
<td>Erythropoiesis</td>
<td>EPO</td>
<td>[84]</td>
</tr>
<tr>
<td>Genetic instability</td>
<td>DEC1, DEC2, MSH2, MSH6</td>
<td>[85-86]</td>
</tr>
<tr>
<td>Glucose metabolism</td>
<td>GLUT1, GPI, HK1, HK2, LDH, MCT1, PGK1, PKM2</td>
<td>[87-92]</td>
</tr>
<tr>
<td>Immortalization</td>
<td>TERT</td>
<td>[93]</td>
</tr>
<tr>
<td>Immune evasion</td>
<td>NTSE</td>
<td>[94]</td>
</tr>
<tr>
<td>Invasion</td>
<td>C-MET, EDN-1, FN-1, MMP-2, MMP-14, SDF-1</td>
<td>[75,81,95-98]</td>
</tr>
<tr>
<td>Metastasis</td>
<td>C-MET, CXCR4, LOX, TWIST1, ZEB1</td>
<td>[75,85,99-101]</td>
</tr>
<tr>
<td>pH regulation</td>
<td>CA-9, CA-12</td>
<td>[102]</td>
</tr>
<tr>
<td>Proliferation</td>
<td>C-MYC, ID2, IGF-2, NOS.</td>
<td>[76,78,103,104]</td>
</tr>
<tr>
<td>Stem cell maintenance</td>
<td>ABCG2, JARID1B, OCT4, NANOG, Sox2, KLF4, c-myc, miR302</td>
<td>[55,65-67,105-107]</td>
</tr>
</tbody>
</table>
Table 2
Small anticancer molecules with hypoxia inducible factor-1 inhibitory activity

<table>
<thead>
<tr>
<th>Molecule</th>
<th>Structure</th>
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</thead>
<tbody>
<tr>
<td>Perfosine</td>
<td><img src="image1" alt="Perfosine structure" /></td>
</tr>
<tr>
<td>Camptothecin</td>
<td><img src="image2" alt="Camptothecin structure" /></td>
</tr>
<tr>
<td>Miltefosine</td>
<td><img src="image3" alt="Miltefosine structure" /></td>
</tr>
<tr>
<td>Topotecan</td>
<td><img src="image4" alt="Topotecan structure" /></td>
</tr>
<tr>
<td>Irinotecan</td>
<td><img src="image5" alt="Irinotecan structure" /></td>
</tr>
<tr>
<td>2-methoxyestradiol</td>
<td><img src="image6" alt="2-methoxyestradiol structure" /></td>
</tr>
<tr>
<td>Temsirolimus</td>
<td><img src="image7" alt="Temsirolimus structure" /></td>
</tr>
<tr>
<td>ENMD-1198</td>
<td><img src="image8" alt="ENMD-1198 structure" /></td>
</tr>
<tr>
<td>Echinomycin</td>
<td><img src="image9" alt="Echinomycin structure" /></td>
</tr>
<tr>
<td>Romidepsin</td>
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</tr>
<tr>
<td>Bortezomb</td>
<td><img src="image11" alt="Bortezomb structure" /></td>
</tr>
</tbody>
</table>

R = OMe, Geldanamycin
R = N-aminooxylyl, 17-AAG
R = Dimethylaminoethylamino, 17-DMAG