Evaluation of HIF-1 inhibitors as anticancer agents

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Hypoxia-inducible factor 1 (HIF-1) regulates the transcription of many genes involved in key aspects of cancer biology, including immortalization, maintenance of stem cell pools, cellular dedifferentiation, genetic instability, vascularization, metabolic reprogramming, autocrine growth factor signaling, invasion/metastasis, and treatment failure. In animal models, HIF-1 overexpression is associated with increased tumor growth, vascularization, and metastasis, whereas HIF-1 loss-of-function has the opposite effect, thus validating HIF-1 as a target. In further support of this conclusion, immunohistochemical detection of HIF-1α overexpression in biopsy sections is a prognostic factor in many cancers. A growing number of novel anticancer agents have been shown to inhibit HIF-1 through a variety of molecular mechanisms. Determining which combination of drugs to administer to any given patient remains a major obstacle to improving cancer treatment outcomes.

Intratumoral hypoxia

The majority of locally advanced solid tumors contain regions of reduced oxygen availability [1]. Intratumoral hypoxia results when cells are located too far from a functional blood vessel for diffusion of adequate amounts of O_2 as a result of rapid cancer cell proliferation and the formation of blood vessels that are structurally and functionally abnormal. In the most extreme case, O_2 concentrations are below those required for survival, resulting in cell death and establishing a selection for cancer cells in which apoptotic pathways are inactivated, anti-apoptotic pathways are activated, or invasion/metastasis pathways that promote escape from the hypoxic microenvironment are activated. This hypoxic adaptation may arise by alterations in gene expression or by mutations in the genome or both and is associated with reduced patient survival [1].

Hypoxia-inducible factor 1 (HIF-1)

The expression of hundreds of genes is altered in each cell exposed to hypoxia [2,3]. Many of these genes are regulated by HIF-1. HIF-1 is a heterodimer formed by the association of an O_2-regulated HIF-1α subunit with a constitutively expressed HIF-1β subunit [4]. The structurally and functionally related HIF-2α protein also dimerizes with HIF-1β and regulates an overlapping battery of target genes [5,6]. Under nonhypoxic conditions, HIF-1α (as well as HIF-2α) is subject to O_2-dependent prolyl hydroxylation [7–9] and this modification is required for binding of the von Hippel–Lindau tumor suppressor protein (VHL), which also binds to Elongin C and thereby recruits a ubiquitin ligase complex that targets HIF-1α for ubiquitination and proteasomal degradation [10,11]. Under hypoxic conditions, the rate of hydroxylation and ubiquitination declines, resulting in accumulation of HIF-1α [12]. Immunohistochemical analysis of tumor biopsies has revealed high levels of HIF-1α in hypoxic but viable tumor cells surrounding areas of necrosis [13,14].

Genetic alterations in cancer cells increase HIF-1 activity

In the majority of clear-cell renal carcinomas, VHL function is lost, resulting in constitutive activation of HIF-1 [11]. After re-introduction of functional VHL, renal carcinoma cell lines are no longer tumorigenic, but can be made tumorigenic by expression of HIF-2α in which the prolyl residues that are subject to hydroxylation have been mutated [15,16]. In addition to VHL loss-of-function, many other genetic alterations that inactivate tumor suppressors

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or activate oncoproteins have been shown to increase the HIF-1 activity in cancer cells through a variety of molecular mechanisms [20]. In general, these changes serve to increase the basal levels of HIF-1α in cancer cells, onto which is superimposed the physiological response to hypoxia.

Significance of HIF-1α overexpression

Independent of any specific mechanism, HIF-1α overexpression is associated with increased patient mortality in many different cancers (Table 1). The basis for this association is the regulation by HIF-1 of genes that play critical roles in many key aspects of cancer pathogenesis, including: immortalization, maintenance of stem cell pools, cellular dedifferentiation, genetic instability, vascularization, metabolic reprogramming, autocrine growth factor signaling, invasion/metastasis, and treatment failure (Table 2). The validation of HIF-1 as a therapeutic target in cancer is based on studies in which genetic manipulations that increase the expression of HIF-1α or HIF-2α in human cancer cells has been shown to increase tumor growth, angiogenesis, and metastasis, whereas genetic manipulations that decrease HIF-1α or HIF-2α expression result in decreased tumor growth, angiogenesis, and metastasis in animal models [17,18,58,96,111].

It should be noted, however, that among both immunohistochemical analyses and animal studies, there are exceptions in which HIF-1α or HIF-2α overexpression is associated with increased patient survival [19,21,22]. In some of these cases, the induction of proapoptotic genes by HIF-1 such as BNIP3 and NIX [23,24] may play an important role in promoting patient survival. In such cancers, however, there may be a strong selection for the eventual emergence of clones with increased activity of anti-apoptotic proteins such as BCL2 [25] or decreased activity of proapoptotic proteins such as p53 [26] that counteract proapoptotic consequences of HIF-1α overexpression, while leaving intact other important properties of HIF-1 such as its ability to promote angiogenesis and invasion. In clear-cell renal carcinoma, decreased expression of the proapoptotic BCL2 family members Bik and Bim [27] and decreased p53 activity [28] may contribute to the resistance of these cells to apoptosis.

TABLE 1

Clinical consequences of HIF-1α (or HIF-2α) overexpression in human cancers, by organ

Cancer	Association[a]	Refs
Bladder	Mortality, MVD[b], tumor grade, TTP	[36,37]
Brain	Mortality, MVD, tumor grade	[35,38,61]
Breast	Mortality, MVD, tumor grade, metastasis	[39–46]
Cervix (uterus)	Mortality, MVD, radiation resistance	[47–49]
Colon	Invasion, metastasis, MVD	[50]
Endometrium (uterus)	Mortality, MVD	[52]
Esophagus	MVD, venous invasion, PDT response[c]	[25,51]
Head and neck	Survival	[21,55]
Head and neck	Mortality (HIF-2α), MVD	[54]
Liver	Venous invasion, MVD	[56]
Lung (NSCLC)	Survival, apoptosis	[57]
Lung (NSCLC)	Mortality	[59]
Oropharynx	Mortality, radiation resistance	[62]
Ovary	Mortality[d], MVD	[26]
Pancreas	Metastasis, MVD, TNM stage	[63,64]
Skin (melanoma)	Mortality (HIF-2α)	[60]
Stomach (GIST)	Mortality, metastasis, MVD	[53]

[a] HIF-1α overexpression is positively associated with indicated parameter unless otherwise noted (HIF-2α) for association with HIF-2α overexpression.
[b] Abbreviations: MVD, microvessel density; TTP, time to progression; MVD (DCIS), microvessel density in ductal carcinoma *in situ*; PDT, photodynamic therapy; GIST, gastrointestinal stromal cell tumor; NSCLC, non-small-cell lung carcinoma; TNM, tumor-node-metastasis.
[c] Tumors with overexpression of both HIF-1α and BCL2.
[d] Tumors with both HIF-1α overexpression and mutant p53.

TABLE 2

Pathophysiological consequences of HIF-1 activation in human cancer cells

Phenotype	Selected HIF-1 target(s)[a]	Refs
Immortalization	Telomerase	[65,66]
	PGM; GPI	[67]
Stem cells	OCT4	[68,128]
Dedifferentiation	ID2	[69]
Genetic instability	MSH2; MSH6	[70,129]
Vascularization	VEGF	[17,71,127]
Metabolism	Glucose transporters; glycolytic enzymes	[71–73,130]
	Pyruvate dehydrogenase kinase 1	[74,75,131]
	Carbonic anhydrase IX	[132]
	Repression of C-MYC/ mitochondrial biogenesis	[76]
Autocrine growth	IGF2; TGF-α	[77,78]
Invasion/metastasis	UPAR; C-MET	[79,80,133]
	Repression of E-cadherin (by ZFHX1B)	[81,82]
	LOX	[83,134]
Treatment failure	ABCB1, ABCG2	[84,85,135]

[a] The listed genes are illustrative not exhaustive. Abbreviations: PGM, phosphoglycerate mutase; GPI, glucosephosphate isomerase; OCT, octamer-binding protein; ID, inhibitor of differentiation; MSH, MutS homolog; VEGF, vascular endothelial growth factor; IGF, insulin-like growth factor; TGF, transforming growth factor; UPAR, urokinase-type plasminogen activator receptor; ZFHX, zinc finger and homeodomain protein; LOX, lysyl oxidase; ABC, ATP-binding cassette transporter protein.

Anticancer agents that inhibit HIF-1

A growing number of anticancer agents have been shown to inhibit HIF-1 activity. For many of these, the mechanism of action has been established and involves a reduction in HIF-1α mRNA or protein levels, HIF-1 DNA-binding activity, or HIF-1-mediated transactivation of target genes (Table 3). Agents that reduce HIF-1α protein levels do so by decreasing the rate of HIF-1α synthesis, increasing the rate of HIF-1α degradation, or both.

In many cancers, mTOR activity is a major determinant of the rate of HIF-1α protein synthesis. The constitutive activation of receptor tyrosine kinases (such as HER2^{neu}, BCR-ABL, and EGFR) and/or the downstream phosphatidylinositol 3-kinase/AKT and RAS/MAP kinase signal transduction pathways in cancer cells leads to increased mTOR activity and the induction of HIF-1 activity [20,86] and the downstream effects on cancer pathogenesis that are described above. Thus, inhibitors of these pathways result in

TABLE 3

Anticancer agents that inhibit HIF-1 activity, by mechanism of action and drug target

I. Decreased HIF-1α mRNA levels: GL331 [100]
II. Decreased HIF-1α protein levels
A. PI3K-AKT-mTOR and RAS-RAF-MEK-ERK pathways
1. BCR-ABL: imatinib/Gleevec [89]
2. EGFR: gefitinib/Iressa, erlotinib/Tarceva, cetuximab/C225 [88]
3. HER2^{neu}: trastuzumab/Herceptin [86,87]
4. mTOR: temsirolimus/CCI-779, everolimus/RAD-001 [86,94,95]
5. Farnesyltransferase: SCH66336 [101]
6. Tyrosine kinase (nonspecific): genistein [109]
7. MEK: PD98059 [104]
8. COX2: NS398, ibuprofen, celecoxib [90]
B. Topoisomerases
1. Topoisomerase I: topotecan [91]
2. Topoisomerase II: NSC 644221 [112]
C. Cyclin-dependent kinases: flavopiridol [99]
D. Microtubule targeting agents: 2-methoxyestradiol, epothilone B, taxotere [97,121]
E. HSP90: 17-AAG, 17-DMAG, apigenin [30,31,110]
F. Histone deacetylases: LAQ824, FK228 [102,103]
G. Thioredoxin: 1-methylpropyl-2-imidazolyl-disulfide, pleurotin [105]
H. Unknown targets: YC-1, PX-478, berberine, pseudolaric acid B, bisphenol A, manassantin B_1, manassantin A, 4-*O*-methylsaucerneol, laurenditerpenol, 103D5R [107,108,113–118]
III. Decreased binding of HIF-1 to DNA: echinomycin, polyamides, DJ12 [98,119,120]
IV. Decreased HIF-1-mediated transactivation
A. Proteasome: bortezomib [92]
B. Histone deacetylases: SAHA/vorinostat, trichostatin A [136]
C. P300: chetomin [106]
D. Unknown: amphotericin B [93]

loss of HIF-1 activity and biological consequences such as impaired tumor vascularization that may contribute significantly to their therapeutic effect.

mTOR regulates the synthesis of many proteins that are important for cancer progression and thus the therapeutic effect of mTOR inhibitors cannot be attributed solely to their inhibition of HIF-1α synthesis. That being said, it appears that in some cancers HIF-1 represents a particularly important target for regulation by mTOR. In a transgenic mouse model of prostatic intraepithelial neoplasia due to prostate-specific expression of activated AKT, the growth of neoplastic lesions could be blocked by treatment with the mTOR inhibitor RAD-001 and microarray analysis of gene expression revealed that, of all the mRNAs assayed, a group encoded by HIF-1 target genes was most significantly upregulated in transgenic compared to nontransgenic prostate tissue and down-regulated in RAD-001-treated compared to untreated transgenic prostate [94]. In renal cell carcinoma, VHL loss-of-function sensitizes the cells to growth arrest by the mTOR inhibitor CCI-779 [95], which is correlated with inhibition of HIF-1α mRNA translation and can be overcome by transfection of an expression vector encoding HIF-1α mRNA lacking the 5′-untranslated region through which mTOR has been shown to regulate HIF-1α protein synthesis [86].

Inhibitors of topoisomerases, cyclin-dependent kinases, and microtubule assembly, heat shock protein 90 (HSP90), histone deacetylases, and thioredoxin also inhibit HIF-1α protein expression, although the mechanisms have not been fully delineated, with the exception of HSP90 inhibitors, which induce HIF-1α degradation, as described below. Other novel HIF-1 inhibitors that induce the degradation of HIF-1α protein have been identified, such as PX-478 and YC-1, but their detailed mechanisms of action remain to be established [29]. Several natural products, including berberine, pseudolaric acid B, bisphenol A, manassantin B_1, manassantin A, 4-*O*-methylsaucernol, and laurenditerpinol, have been shown to inhibit HIF-1α protein expression but neither their mechanism of action nor their anticancer effects *in vivo* have been reported.

The chaperone HSP90 interacts with HIF-1α and is required for HIF-1 transcriptional activity. Inhibitors of HSP90 such as geldanamycin and its derivatives 17-allylamino-17-demethoxygeldanamycin (17-AAG) and 17-dimethylaminomethylamino-17-demethoxygeldanamycin (17-DMAG) induce the ubiquitination and proteasomal degradation of HIF-1α even in the absence of VHL [30]. The receptor for activated C kinase 1 (RACK1), which was originally identified as an anchoring protein for activated protein kinase C but is now recognized as a multifunctional scaffold protein that plays an important role in diverse biological processes, competes with HSP90 for binding to HIF-1α [31]. HSP90 inhibition by 17-AAG leads to unopposed RACK1 binding to HIF-1α, which triggers ubiquitination and proteasomal degradation. RACK1 binds to Elongin C via an amino acid sequence with striking similarity to the region of VHL that interacts with Elongin C. Thus, RACK1 recruits an ubiquitin ligase complex similar to that which is recruited by VHL, establishing a parallel but O_2-independent pathway for the proteasomal degradation of HIF-1α [31]. HSP90 inhibitors may be particularly potent anticancer agents because in addition to its role in promoting HIF-1α stability, HSP90 is also required to prevent the degradation of many activated or overexpressed oncoproteins, including receptor tyrosine kinases and serine/threonine kinases [139]. Both 17-AAG and 17-DMAG are currently in clinical trials.

Other HIF-1 inhibitors do not affect HIF-1α mRNA or protein levels but prevent HIF-1 from activating transcription of target genes. Echinomycin [98], polyamides [119], and DJ12 [120] accomplish this by blocking the binding of HIF-1 to DNA. Echinomycin preferentially binds to the DNA sequences 5′-ACGT-3′ and 5′-TCGT-3′ [98] and thus will compete with HIF-1, which binds at sites containing the core sequences 5′-ACGTG-3′ or 5′-GCGTG-3′ [126], the latter of which is not a high-affinity site for echinomycin binding. Given the limited sequence specificity of echinomycin, it is likely to inhibit the DNA binding of other transcription factors including C-MYC, which binds to the sequence 5′-CACGTG-3′ [98].

Another mechanism by which HIF-1 is inhibited is at the level of transactivation. The proteasome inhibitor bortezomib has the paradoxical effect of increasing the levels of HIF-1α protein by blocking its degradation while at the same time interfering with the function of the carboxyl-terminal transactivation domain (TAD-C) of HIF-1α [92]. The activity of TAD-C has been attributed

to the recruitment of the coactivators p300 and CBP, which bind to HIF-1α under hypoxic conditions, whereas under normoxic conditions, the O_2-dependent hydroxylation of asparagine residue 803 by factor inhibiting HIF-1 (FIH-1) blocks this interaction [122]. Bortezomib was found to have no effect on the interaction of HIF-1α with p300, so the molecular mechanism by which bortezomib blocks HIF-1-mediated transcription remains to be established [92]. The antifungal drug amphotericin B also inhibits HIF-1 transcription through an effect on TAD-C by promoting the interaction of HIF-1α with FIH-1, leading to increased asparaginyl hydroxylation and decreased recruitment of p300 [93]. At concentrations lower than those required to induce the degradation of HIF-1α, the histone deacetylase inhibitors suberoylanilide hydroxamic acid (SAHA) and trichostatin A inhibit TAD-C function and p300 interaction by a mechanism that is independent of asparaginyl hydroxylation [136].

HIF-1 activity is also critical for the function of stromal cells that play important roles in tumorigenesis, including endothelial cells and macrophages [123,124]. HIF-1 regulates hundreds of genes in vascular endothelial cells [2] and one of the major effects of treating endothelial cells with the novel anti-angiogenic agent endostatin is the inhibition of HIF-1-dependent gene transcription [125]. Although the molecular mechanisms underlying this observation remain to be established, it appears that the anticancer effects of HIF-1 inhibitors may result from effects on both cancer cells and stromal cells.

Caveat emptor

Although there is a large body of data linking HIF-1 to the expression of specific target genes that promote key aspects of cancer progression, there are several important caveats that must be appreciated. First, among the many hundreds of genes that are potentially regulated by HIF-1, only a subset of these are under the control of HIF-1 in any given normal or cancer cell [2,6,32]. Worse yet, in response to hypoxia or HIF-1α overexpression, the expression of some HIF-1-regulated genes has been found to increase, decrease, or remain unchanged depending upon the particular cell type that is analyzed [32]. This may be due in part to whether HIF-2α is expressed in the cell in addition to HIF-1α, as HIF-1α and HIF-2α may have opposite effects on the expression of some genes [33]. Second, although HIF-1 induces the expression of many genes that promote tumor cell survival, it also can induce the expression of genes that promote growth arrest [33] or tumor cell death [34]. To the extent that such gene products are expressed and their activities not counteracted by other coexpressed proteins, HIF-1α overexpression may be associated with increased patient survival.

As a result of the complexity of the HIF-1 transcriptome, the consequences of increasing or decreasing HIF-1 activity within a given cancer cell cannot be predicted *a priori* because the net effect of the particular target genes activated, although generally favoring cancer progression, may in some cases impair cancer cell proliferation/survival. Even if the entire battery of HIF-1-regulated genes in the cancer cell could be identified, it would still not be possible to predict with certainty the consequences of altering HIF-1 activity. For example, expression of BNIP3 mRNA may be irrelevant if it is transcribed from a gene containing a mutation that eliminates the expression of a functional protein. Alternatively, overexpression of anti-apoptotic BCL2 family members may negate the effect of BNIP3 induction, thus favoring the positive effects of HIF-1 on tumor metabolism and vascularization. This dynamic heterogeneity is of course the major obstacle to all efforts to develop effective cancer therapies. Finally, genetic manipulations that alter HIF-1 activity in cancer cells may not provide an accurate assessment of the effect of HIF-1 inhibitors, since the latter will also block HIF-1 in stromal cells, which play important roles in angiogenesis and other key aspects of cancer biology. It is important to note that the increase in tumor growth observed in some experimental models when HIF-1 activity is knocked down genetically has not been observed in studies involving administration of small molecule inhibitors of HIF-1.

Combination therapy

Because of the powerful selection that results in the eventual emergence of cells resistant to any known anticancer agent, the administration of multiple agents simultaneously is essential for the successful treatment of human cancer. Successful treatment of tuberculosis requires the administration of three antibiotics; successful treatment of AIDS requires the administration of three antiviral agents; and it is not reasonable to expect that the successful treatment of cancer can be accomplished reliably with any fewer than three anticancer agents. Among patients with tumor recurrence following treatment with Gleevec, mutations that confer resistance to Gleevec were identified [141]. That this predictable finding appeared in a high impact journal illustrates the continued lack of understanding of the power of mutation and selection in human cancer. Thus, the use of HIF-1 inhibitors as anticancer agents must occur within the conceptual framework of combination therapy. A simple paradigm to consider is the following general pathway:

(I) oncogene gain-of-function → activation of signal transduction pathway(s) → HIF-1 activation → downstream gene expression → pathogenic response.

A specific example is the following common scenario in breast adenocarcinoma:

(II) $HER2^{neu}$ gene amplification → PI3K/AKT/mTOR signaling → HIF-1 activation → expression of VEGF and other angiogenic cytokines → tumor vascularization.

If we set as our goal the maximal reduction possible in tumor vascularization with the minimal risk of emergence of a resistant clone, then it stands to reason that this can best be accomplished by targeting pathway (II) at multiple sites with some combination of available inhibitors targeting $HER2^{neu}$, mTOR, HIF-1, and VEGF/VEGFR. We currently possess the knowledge of pathways and some of the requisite targeted agents to test this hypothesis.

Efforts have also been made to investigate whether angiogenesis inhibitors can improve outcome in cancers that are treated by radiation therapy [140]. Studies from animal models indicate that reoxygenation of hypoxic cancer cells following radiation results in the production of reactive oxygen species that induce HIF-1 activity, leading to the production of VEGF and FGF2, which bind to their cognate receptors on vascular endothelial cells and prevent radiation-induced apoptosis [135]. However, when animals were treated with the angiogenesis inhibitor canstatin, the radiation-induced HIF-1 activity resulted in tumor apoptosis rather than radiation resistance [137]. These findings illustrate the benefits of combined radiation and drug therapy and suggest that, in

some cancers, elevated HIF-1 activity may be exploited to kill cancer cells [138].

Conclusion

The greatest obstacle to the establishment of efficacious therapies for human cancer is the heterogeneity of the disease within a single individual over time and space, as well as the heterogeneity that exists between individuals with the same type of cancer. Although cancer research has demonstrated that there are hundreds of genetic and epigenetic alterations in cancer cells, understanding which of these changes represent critical therapeutic targets in a given patient is still a major challenge. Because HIF-1 controls the expression of so many genes that impact on cancer progression, both in tumor and stromal cells, it may be an important target for cancer therapy. However, as for most other candidate drug targets, successful translation of basic science research into clinical applications will require new methods for establishing the proper context for administration of HIF-1 inhibitors, that is selection of an appropriate patient cohort and multidrug treatment regimen.

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