Hypoxia inducible factor-1: a novel target for cancer therapy

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Hypoxia develops in the majority of solid tumors due to the inability of the existing vascular system to supply the growing tumor mass with adequate amounts of oxygen. A large body of clinical evidence suggests that intratumoral hypoxia correlates with the elevated aggressive behavior of cancer cells and their resistance to therapy, leading to poor patient prognoses. A heterodimeric transcription factor, hypoxia inducible factor-1 (HIF-1), has been shown to orchestrate a large number of molecular events required for the adaptation of tumor cells to hypoxia. Therefore, HIF-1 has become an attractive target for the development of anti-cancer drugs. Here, we highlight some of the recently developed small-molecule inhibitors of HIF-1 function. These drugs disrupt the HIF-1 signaling pathway through a variety of mechanisms, including the inhibition of HIF-1α protein synthesis, stabilization, nuclear translocation and HIF-1 transactivation of target genes. *Anti-Cancer Drugs* **16:901–909 © 2005 Lippincott Williams & Wilkins.**

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Hypoxia and cancer

Targeted therapeutic agents are a relatively recent addition to the arsenal of anti-cancer drugs. These molecules are designed to disrupt molecular pathways that are critical to the survival of tumor cells, but are either inactive or dispensable in normal cells. Clinical success of such drugs as imatinib (Bcr–Abl), and erlotinib and gefitinib (epidermal growth factor receptor) demonstrates the validity of targeted approaches and encourages the search for new molecular targets [1].

Hypoxia, a condition of oxygen deprivation, is a common feature of many forms of solid tumors [2–4]. It emerges when the stromal vascular system fails to deliver a sufficient amount of oxygen to the growing tumor (Fig. 1). As a result, many tumors undergo adaptive genetic changes that allow them to avoid hypoxia-induced cell death and necrosis. Such adaptation also makes tumors more aggressive and confers resistance to conventional therapy. For example, successful radiotherapy relies on the generation of oxygen radicals in order to destroy tumor tissue, thus allowing low-oxygen or hypoxic areas to escape major damage. Chemotherapy is also less effective in the areas of hypoxia. First, the diffusion of drugs is greatly attenuated due to the large distances between the stromal blood vessels and the hypoxic tumor cells. Second, hypoxic cells exhibit decreased rates of cell division and most drugs target dividing cells. Hypoxic cells also exhibit increased expression of drug-resistance genes, such as MDR1 and P-glycoprotein. Finally, hypoxia drives genetic selection of cells that are resistant to p53-mediated apoptosis, making tumors less prone to drug-induced cell death [5]. Collectively, these factors explain why the development of intratumoral hypoxia strongly correlates with the unfavorable outcome of cancer treatment [6].

Hypoxia is a major driving force of tumor progression in over 70% of human cancers including those of breast [7], prostate [8], cervix [9], ovary, oropharynx [10], head and neck [11], lung [12], and brain [3,13,14]. In recent years, considerable research effort, both in academic and industrial sectors, has focused on the development of therapies that specifically target hypoxic cells. In addition, several clinically used agents have been evaluated for their activity against hypoxic cells.

Molecular biology of the hypoxic response

Many events that occur during the adaptation of a tumor to hypoxia are mediated through a group of hypoxia inducible factors (HIFs), of which HIF-1 is the most significant and best studied [15,16]. HIF-1 is a heterodimeric transcription factor, consisting of an oxygen-sensitive HIF-1α subunit and a constitutively present HIF-1β subunit [also known as aryl hydrocarbon nuclear translocator (ARNT)]. The two proteins have similar domain structure. Both contain a basic helix–loop–helix domain required for their dimerization and DNA binding, a Per-ARNT-Sim domain (PAS) that is also involved in dimer formation and transactivation domains (TAD). The TAD domain of HIF-1α has been shown to bind co-activator proteins p300/CBP, SRC-1 and TIF2, whereas the TAD of HIF-1β appears to be dispensable for the activity of the HIF-1 complex. In addition, HIF-1α

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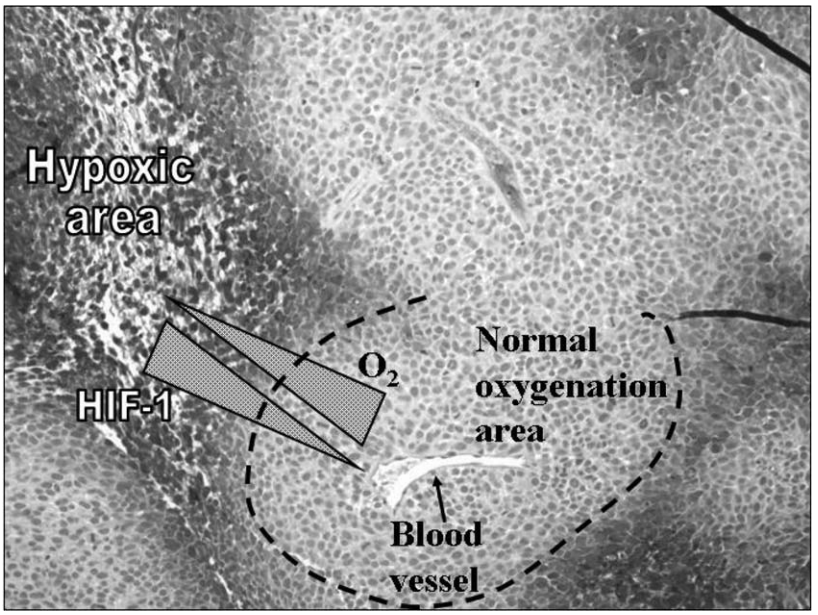
Fig. 1



Visualization of intratumoral hypoxia. A section of a s.c. grown xenograft of LN-229 human glioma was stained with an oxygen-sensitive reagent, pimonidazole. Normal oxygenation area (inside the dashed line) extends to a distance of approximately 10 cell layers from the blood vessel. Dark staining indicates the areas of hypoxia. Decreasing oxygen concentration coincides with the increasing levels of the HIF-1 transcription factor [4].

contains an oxygen-dependent degradation domain between residues 401 and 603. This domain contains several amino acid residues that target HIF-1α for proteosomal degradation under normoxic conditions.

The *HIF-1α* gene promoter contains recognition sites that bind several ubiquitous transcriptional activators such as Sp-1, AP-1, AP-2 and NF-1, causing the gene to be constitutively expressed. The levels of HIF-1α are largely regulated on a post-transcriptional level, by the rate of protein synthesis and degradation. Under normal oxygen conditions, HIF-1α is hydroxylated at proline residues 402 and 564 by three prolyl hydroxylases, PHD1–3 [17]. These modifications allow binding to the tumor suppressor protein Von Hippel–Lindau (pVHL), a recognition component of an E3 ubiquitin ligase. This leads to HIF-1α ubiquitylation, which mediates its rapid proteosomal degradation [18]. In addition, HIF-1α is acetylated at Lys532 by the ARD1 acetyltransferase [19]. Although this modification also facilitates recognition of HIF-1α by pVHL, the activity of ARD1 does not appear to be dependent on oxygen concentration. Another mechanism of HIF-1 regulation prevents its transcriptional activity under normoxia. Factor inhibiting HIF-1 (FIH-1) is an oxygen-dependent enzyme that hydroxylates Asn803 within the TAD of HIF-1α, disrupting its interaction with the transcription co-activators p300 and CBP [20].

Under hypoxic conditions these hydroxylation steps are inhibited, slowing the rate of HIF-1α degradation and allowing large quantities of the protein to accumulate in the cell. Although the global protein translation rate is significantly reduced in cells during hypoxic stress, the rate of HIF-1α protein synthesis does not appear to be compromised. This may be related to the presence of an efficient internal ribosome entry site (IRES) in the HIF-1α mRNA, making its translation cap-independent during hypoxia [21]. To become functional HIF-1α needs to translocate into the nucleus where it will bind to HIF-1β and co-activator proteins. The resulting complex forms a functional transcription factor that can bind to hypoxia response elements (HRE) with the consensus DNA sequence 5′-RCGTG-3′ [22] in the promoter and enhancer regions of over 70 human genes, and activates their expression.

Two broad classes of HIF-1 target genes contribute to tumor survival and progression. The first class consists of the genes that allow short-term survival of oxygen-starved cells by switching the cell's energy generating metabolism from oxidative phosphorylation to anaerobic glycolysis. The second class of HIF-1 target genes ensures the long-term sustainability of the tumor cell mass by inducing the formation of new blood vessels [4]. Several examples of these genes are presented in Table 1.

Table 1 Major classes of HIF-1 target genes

Short-term adaptation		Long-term survival and proliferation	
Function	Gene	Function	Gene
Glucose transport	Glut-1	angiogenesis	VEGF
Glucose metabolism	GAPDH		TGF-β3
	enolase 1	cell survival	EPO
	hexokinase 1		ADM
	LDHA	cell proliferation	TGF-α
	PFKBF3		IGF2
Amino acid metabolism	transglutaminase 2	drug resistance	MDR1
Iron homeostasis	transferrin		P-glycoprotein
Nucleotide metabolism	adenylate kinase 3	invasion	MMP2
pH regulation	carbonic anhydrase 9		UPAR

Glut-1, glucose transporter 1; GAPDH, glyceraldehyde-3-P-dehydrogenase; LDHA, lactate dehydrogenase A; PFKBF3, 6-phosphofructo-2-kinase/fructose-2,6-biphosphatase-3; VEGF, vascular endothelial growth factor; TGF-β3, transforming growth factor-β3; EPO, erythropoietin; ADM, adrenomedullin; TGF-α, transforming growth factor-α; IGF2, insulin-like growth factor 2; MDR1, multidrug resistance 1; MMP2, matrix metalloproteinase 2; UPAR, urokinase plasminogen activator receptor.

Under normal oxygen concentrations, HIF-1 can be induced by a variety of stimuli, such as growth factors, cytokines, hormones and viral proteins. Phosphatidylinositol 3-kinase (PI3K) [23,24] and MEK1/ERK pathways are known to mediate the accumulation of HIF-1 in normoxic cells. These pathways are often upregulated in tumor cells due to the activation of oncogenes and loss of tumor suppressors, notably PTEN, pVHL and p53 [49].

Pharmacological targeting of HIF-1

Given the central role of hypoxia and HIF-1 in the activation of numerous pathways responsible for tumor progression, it is not surprising that targeting hypoxic cells has become an attractive therapeutic strategy that is being actively pursued [25,26]. Disruption of hypoxic pathways is expected to cause tumor cell death due to metabolic dysregulation and reduced formation of blood vessels. The following general strategies have been used to target hypoxic cells: (i) inhibition of HIF-1 by small-molecule therapeutics and antisense approaches [27,28], and (ii) design of hypoxia-dependent cytotoxic drugs [29] and hypoxia-responsive viral vectors [30,31]. Here, we highlight some of the recent advances in pharmacological targeting of HIF-1. The discussion is limited to the inhibitors of the molecular pathways shown in Fig. 2. Further overview of HIF-1 as a cancer drug target can be found elsewhere [32,33].

Topotecan

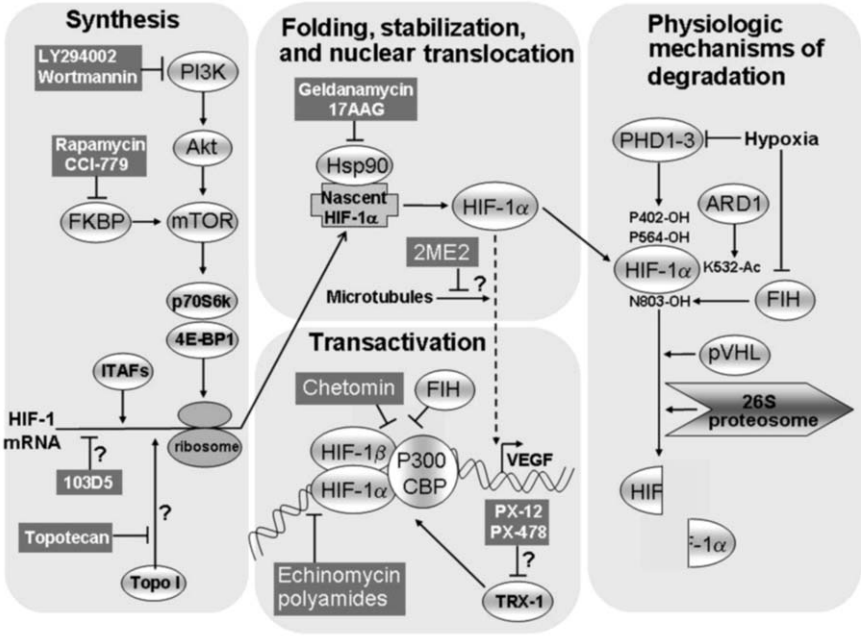
Cell-based reporter assays have been used for the rapid screening of large chemical libraries to identify agents that inhibit HIF-1 transcriptional activity [34,35]. In the first study, U251MG human glioma cells were stably transfected with a luciferase reporter construct regulated by canonical HREs. A'diversity set' of approximately 2000 compounds, representative of the three-dimensional structures in a larger National Cancer Institute library, was screened to identify compounds that attenuated hypoxia-induced luciferase expression. One of the compounds identified in the screen was topotecan (NSC-609699), a topoisomerase I inhibitor with known anti-cancer activity [34]. It inhibited hypoxia-induced accumulation of HIF-1α in cell culture at a low nanomolar concentration ($EC_{50} = 54$ nM). The ability of topotecan to inhibit HIF-1 was tested in a s.c. U251MG glioma xenograft model. Daily, low-dose administration (1 mg/kg topotecan, qd × 10) led to a significant reduction of large preformed tumors. A decrease in tumor cellularity and an increase in deposition of extracellular matrix components were also observed in treated animals. Importantly, these changes accompanied a dramatic reduction of HIF-1α protein level and the level of its target gene products. Tumor levels of human VEGF and phosphoglycerate kinase 1 mRNAs decreased by 95 and 90%, respectively. These results suggest that topotecan may exert some of its anti-tumor properties via inhibition of one of the HIF-1 activation pathways. Indeed, a recent study demonstrated that topotecan treatment inhibits HIF-1α translation through a topoisomerase I-dependent mechanism, but does not affect HIF-1α gene transcription or HIF-1α protein half-life [36].

Topotecan chemotherapy is currently used in the clinic to treat small cell lung cancer and ovarian cancer. Its cytotoxic activity in cancer cells is linked to replication-mediated DNA damage. However, a large-dose, intermittent schedule (10 mg/kg, q4d × 3) of topotecan administration did not affect tumor growth in the U251MG glioma xenograft model, suggesting that a metronomic treatment may be critical for efficient HIF-1 targeting. It is possible that changing the schedule may expand the range of applications of this drug.

103D5R

A second study used a similar cell-based assay to screen a combinatorial library of 10 000 natural product-like compounds based on the 2,2-dimethylbenzopyrane motif [35]. The screen was conducted in LN229 human glioblastoma cells stably expressing an alkaline phosphatase reporter gene controlled by six copies of the

Fig. 2



Small-molecule inhibitors of the HIF-1 pathway. Molecular events that contribute to the cellular homeostasis of HIF-1α are grouped into four general classes (shaded areas). HIF-1α inhibitors that affect protein synthesis, folding, nuclear translocation and transactivation function are shown in grey boxes. The mechanism of action and molecular targets are known for some inhibitors, but remain putative for others.

VEGF gene HRE [37]. One of the most potent inhibitors identified in the screen, 103D5R, had an $EC_{50} = 35 \pm 6\,\mu M$. In addition to reducing HIF-1α levels under hypoxia, 103D5R also reduced the phosphorylation of Akt, SAPK/JNK and Erk1/2. These changes occurred after the decrease in the level of HIF-1α, suggesting that the effects of 103D5R on these pathways are unlikely the cause of HIF-1α inhibition. Initial characterization of the mechanism of action of this inhibitor demonstrated that it does not influence the rate of transcription of the *HIF-1α* gene or the half-life of the protein, but rather causes diminished synthesis of the HIF-1α protein. The mechanism by which 103D5 may regulate HIF-1α protein synthesis is currently unknown. Among many possibilities, one attractive hypothesis is that the drug may inhibit the activity of the IRES transacting factors (ITAFs) [38] that are required for efficient translation initiation at the HIF-1α mRNA IRES during hypoxia. Treatment of glioblastoma cells with 103D5R dramatically decreases the expression levels of HIF-1 target genes *VEGF* and *Glut-1*, both of which are associated with the aggressive tumor phenotype. Studies are currently underway to determine the anti-tumor effect of this novel inhibitor in preclinical models.

Cell-based assays, such as the ones described above, are designed to measure transcriptional activity of HIF-1 and, therefore, could identify inhibitors of a wide range of upstream molecular pathways. These inhibitors are likely to exhibit pleiotropic characteristics making it difficult to determine if their anti-cancer effects are exerted via the inhibition of HIF-1 or other molecular targets.

Redox pathway inhibitors: pleurotin, PX-12 and PX-478

Thioredoxin (Trx)-1 is a small redox protein that modulates the activity of a number of signaling molecules, such as protein kinase C, NF-κB and p53, in response to the redox state of the cellular environment. Trx-1 has been shown to also reduce Ref-1, a protein with DNA repair endonuclease and redox regulatory activities. Ref-1 reduces a cysteine residue in the C-terminal TAD of HIF-1, augmenting its transactivating potential [39]. It has been demonstrated that overexpression of Trx-1 leads to increased angiogenesis in tumor xenografts as a result of elevated levels of HIF-1α and VEGF. Conversely, the expression of an inactive mutant of Trx-1 reduces the activity of HIF-1α and VEGF expression levels [40]. Two potent irreversible inhibitors of Trx-1, PX-12 and pleurotin, have been tested for their ability to inhibit HIF-1 in cell culture and xenograft models. Both inhibitors reduced the levels of HIF-1α and its transactivating potential, the levels of VEGF and inducible nitric oxide synthase, and microvessel density in tumors [41].

Another inhibitor that may act through the redox-sensing mechanism is PX-478. The drug decreases cellular levels

of HIF-1α in a VHL and p53-independent manner [42]. The anti-tumor activity of PX-478 was tested in mouse subcutaneous xenograft models corresponding to several aggressive tumor types that are resistant to experimental therapies [43]. The initial tumor size varied between 100 and 410 mm^3. The drug was administered on a q1d × 5 schedule at 60–120 mg/kg/day. Ovarian (OvCar-3), prostate (PC-3 and DU-145), breast (MCF-7), renal (Caki-1), pancreatic (Panc-1, MiaPaCa and BxPC-3) and small cell lung (SHP-77) cancer xenografts were all significantly affected by the treatment. At the higher dose of the drug these tumors underwent 20–100% regression and more than 20-day growth delays. SHP-77 xenograft with an initial volume of 160 mm^3 was completely cured by either 100 or 75 mg/kg/day dosing of PX-478. An important feature of PX-478 is that it appears to be more active on large, well-established tumors than on smaller ones. Given the refractive nature of large tumors to many existing therapies, this characteristic of PX-478 may become extremely beneficial in the clinic. The agent has a favorable toxicity profile in mice, with neutropenia being the major toxic effect, along with weight loss and decreased food intake. These effects were reversible upon the cessation of treatment. No acute liver or renal toxicities were observed.

PX-478 caused a reduction in the levels of the HIF-1α by 71%, of VEGF by 38% and of Glut-1 by 76%. A relatively small effect of the drug on the expression of VEGF compared to a larger and more prolonged decrease in Glut-1 levels suggests that the dysregulation of glucose metabolism, rather than disruption of neovascularization, may be the predominant cause of the pronounced anti-tumor activity of PX-478. This notion is supported by the observed lack of major differences in the degree of vascularity between treated and untreated tumors. Potential HIF-1-independent effects of PX-478 on tumour growth have not been investigated.

Microtubule cytoskeleton inhibitors: 2ME2, vincristine and taxol

In addition to the search for novel drugs that target HIF-1 signaling, several established anti-tumor agents have been evaluated for their ability to inhibit HIF-1.

2ME2 is an orally available, well-tolerated compound that is currently undergoing phase II clinical trials in patients with multiple myeloma, breast, prostate and ovarian cancer [44]. It is a natural metabolite of estrogen that exhibits pleiotropic anti-tumor properties by inducing cancer cell apoptosis and inhibiting angiogenesis. These effects have been attributed to the ability of 2ME2 to interact with the colchicine-binding site in tubulin and cause depolymerization of microtubules. Indeed, in a mouse orthotopic breast tumor model, daily administration of therapeutic quantities of 2ME2 (150 mg/kg) led to the appearance of aberrant mitotic arrest in both tumor and endothelial cells as a result of microtubule depolymerization and multiple mitotic asters. A recent study showed that the anti-angiogenic activity of 2ME2 is likely in part mediated through the HIF-1 pathway and its downstream targets [45]. Treatment of cancer cells with 2ME2 resulted in significant reduction of HIF-1α protein level without affecting the rates of *HIF-1α* gene transcription or HIF-1α proteosomal degradation. The mechanism of 2ME2-mediated effect on the HIF-1α protein is not known, but it may be linked to the role of microtubules in the process of HIF-1α nuclear translocation. Taxol and vincristine, agents that stabilize microtubules, also cause the reduction of HIF-1α protein levels. Elucidation of the role of microtubules in HIF-1α synthesis will likely identify new strategies for the pharmacologic targeting of HIF-1.

Heat shock protein 90 (Hsp90) inhibitors: geldanamycin (GA) and 17-allylamino-17-demethoxygeldanamycin (17-AAG)

GA specifically binds to the ATP-binding pocket in the N-terminus of the molecular chaperone Hsp90 and inhibits its ATPase activity [46]. Hsp90 is involved in the activity of many cancer-related signaling proteins, such as p53, Akt, Bcr–Abl, HIF-1α and others. Treatment of the human PC-3 prostate cancer cell line with GA was shown to destabilize HIF-1α protein and cause its proteosomal degradation without affecting the rates of its transcription or synthesis [47]. The effect of GA on the level of nuclear and cytoplasmic HIF-1α was blocked by the proteasome inhibitors LCN and MG-132. However, preventing proteosomal degradation of HIF-1α did not restore its transcriptional activity in GA-treated cells, suggesting that Hsp90 is also required for the proper folding of HIF-1α. The effect of GA on HIF-1α was also shown to be independent of the PI3K pathway.

GA effectively inhibits HIF-1α in cells derived from a variety of cancers, including prostate, colonic, hepatic, ovarian, brain and breast tumors, all of which develop intratumoral hypoxia. Therefore, the established anti-tumor activity of GA and its chemical derivative 17-AAG [48] are likely to be, at least in part, due to the inhibition of HIF-1 activated targets.

PI3K/Akt/mammalian target of rapamycin (mTOR) pathway inhibitors: wortmannin, LY294002, rapamycin, CCI-779 and Rad001

HIF-1 is present at high levels under normoxic conditions in over 70% of human cancer cell lines, contributing to the aggressive phenotype of the corresponding tumor types. The PI3K/Akt/mTOR signaling pathway plays a major role in HIF-1 upregulation in these cell lines, primarily by increasing the rate of HIF-1α protein translation. Therapeutic inhibition of PI3K by wortmannin and LY294002 attenuates HIF-1α protein synthesis

rate with a minimal effect on the level of gene transcription in osteosarcoma, renal carcinoma, and prostate cancer cell lines [23]. The tumor suppressor PTEN is known to control the activity of Akt. Its loss in human glioblastoma leads to upregulation of Akt activity and over-expression of HIF-1α under normoxic conditions [49].

One of the downstream targets of the PI3K signaling cascade is mTOR. mTOR regulates protein synthesis through phosphorylation of two targets: p70 S6 kinase and translation-repressor protein 4E-BP1 (PHAS-1) [50]. The only known physiologic substrate of p70S6K is the 40S ribosomal protein S6 whose phosphorylation leads to enhanced protein translation. Phosphorylation of 4E-BP1 induces its dissociation from eIF-4E and consequently increases the rate of translation initiation. Thus, both targets of mTOR/p70S6K signaling lead to an increased rate of protein synthesis. A clinically used drug rapamycin [51], and its chemical derivatives CCI-779 and Rad001 [52], are specific inhibitors of mTOR and have been shown to reduce cellular levels of HIF-1α.

Both the inhibitors of PI3K and mTOR are currently in clinical trials as anti-cancer agents (Table 2). Since PI3K signaling affects a wide variety of cellular processes, it will be a major challenge to establish whether and to what extent the anti-tumor activities of these agents are due to the inhibition of HIF-1α.

HIF-1α transactivation: chetomin, echinomycin and polyamide inhibitors

To find more specific HIF-1 inhibitors, several groups focused on the transcriptional activity of HIF-1 as a therapeutic target. One study attempted to identify specific inhibitors of the interaction of HIF-1 with the transcriptional co-activator p300 [53]. Prior studies had shown that disrupting this interaction with retrovirally expressed polypeptides had anti-tumor effects in colon and breast xenograft models [54]. An *in vitro* screening system was developed in which a peptide corresponding to the p300-interacting region of HIF-1α was immobilized on a test plate. A second peptide consisting of the CH1 domain of p300 fused to GST was added and the interaction between the two polypeptides was quantitatively assessed using an anti-GST antibody fluorescently labeled with europium. Screening a library of over 600 000 compounds led to the identification of chetomin, a submicromolar inhibitor of the HIF-1α/p300 interaction. Chetomin binds to the CH1 domain of p300, perturbing its three-dimensional structure and rendering it incapable of interacting with HIF-1α. This effect, however, appears to be limited to the CH1 domain, since the activity of transcription factors (RAR, SREBP2 and SRC-1) that bind to other domains of p300 are unaffected.

The anti-cancer efficacy of chetomin was investigated in the HCT116 colon cancer xenograft model. Mice bearing small s.c. tumors of an average size of 50 mm^3 were given daily chetomin injections at 2 mg/kg. Drug-treated mice showed significant levels of necrosis in tumor tissues compared to the vehicle control. Lowering the dose of chetomin to 1 mg/kg was shown to still produce an anti-tumor effect in PC-3 prostate xenografts. The observed side-effects of chetomin treatment include coagulative necrosis at sites of injection, anemia and leukocytosis. No end-organ toxicity was detected.

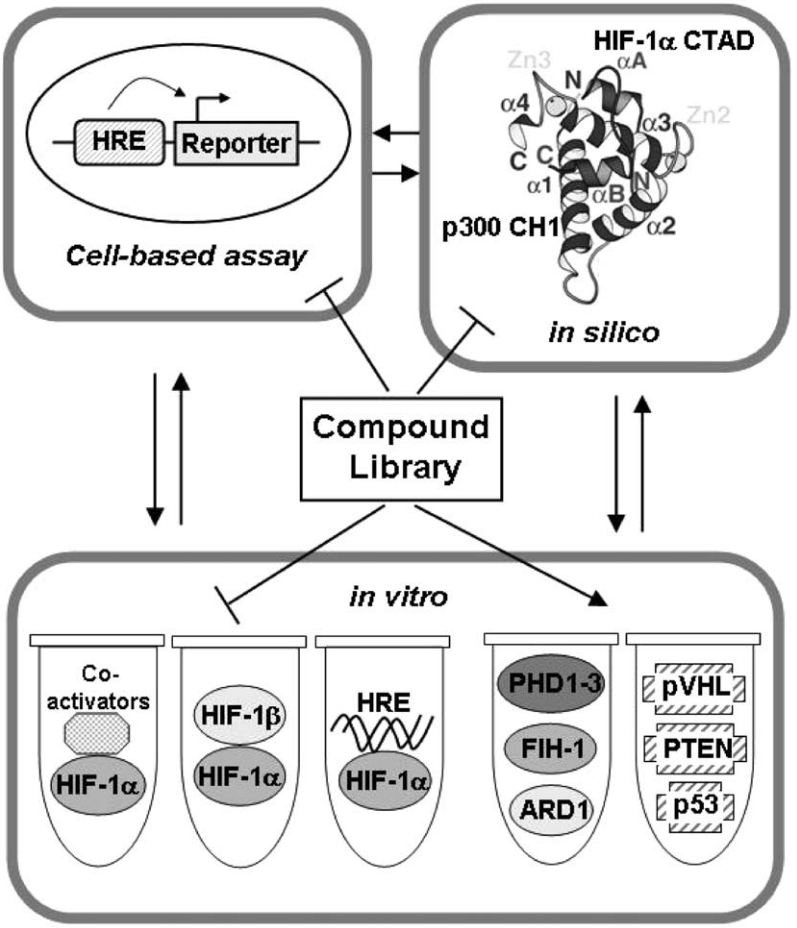
Since the CH1 domain of p300 interacts with a number of additional transcription factors, including STAT2, NF-κB, c-myb, c-Jun, Elk and p53, it was important to show that the observed effects are caused by the inhibition of HIF-1. Indeed, the introduction of a p300-independent form of HIF-1α (HIF-1α-VP16) into the tumor cells partially rescued their growth in the xenografts in chetomin-treated animals.

The interaction of HIF-1 with the HRE was also shown to be a tractable therapeutic target. A fluorescently-labeled *N*-methylpyrrole and *N*-methylimidazole polyamide was designed to specifically bind to the minimal HRE sequence 5′-WTWCGW-3′ from the *VEGF* gene promoter [55]. The molecule was shown to accumulate in the nucleus, and inhibit hypoxia-induced *VEGF* transcription by 60% at 1 μM concentration in cultured cells. The HIF-1–HRE interaction can also be disrupted by a small-molecule drug echinomycin [60]. Echinomycin specifically binds to the core of the HIF-1 recognition sequence 5′-CGTG-3′ and inhibits HRE-mediated reporter activation in U251 glioma cells with an $EC_{50} = 1.2$ nM. Hypoxia-induced expression of the endogenous *VEGF* gene is also inhibited at a low nanomolar concentration. The DNA binding sites of HIF-1α and c-Myc are known to share the 5′-CGTG-3′ core sequence. Therefore, it will be useful to determine the global transcriptional response in echinomycin-treated cells. Finally, it is important to note that the anti-cancer activity of echinomycin has been evaluated in clinical trials in the past. The results of these studies have been disappointing [56,57]. New information on the molecular mechanism of this drug may prove useful in re-evaluating available clinical data.

Conclusion and future directions

Hypoxia, and the HIF-1 transcription factor in particular, have become important and widely pursued targets in the development of small molecule therapeutics. Cell-based and *in vitro* high-throughput screening strategies (see Fig. 3 below) have proven instrumental in identifying drug candidates with promising pharmacology and efficacy profiles. Furthermore, inhibitors of several signaling pathways have been tested for their ability to inhibit HIF-1 and its downstream targets.

Fig. 3



Strategies for the high-throughput identification of HIF-1 inhibitors. Both *in vitro* (lower box) and cell-based assays (upper left box) have been used to screen chemical libraries for HIF-1 inhibitors. Several examples are shown of specific protein–protein and DNA–protein interactions that can be used for inhibitor screening *in vitro* (three tubes on left). An alternative use of *in vitro* assays is to screen for the agonists of the enzymes involved in physiologic degradation of HIF-1α (two tubes on right). The structural information on the HIF-1 transcription activation complex can be used in rational inhibitor design and *in silico* library screening (upper right box). HIF-1α CTAD, the C-terminal activation domain of HIF-1α. For other abbreviations see text.

HIF-1 inhibitors identified to date interfere with the following general mechanisms: (i) control of HIF-1α synthesis, (ii) folding, stabilization and nuclear translocation, and (iii) HIF-1α transactivation of target genes. The inhibitors representing each mechanistic group are highlighted in Fig. 2 and listed in Table 2. None of the presently available inhibitors appears to disrupt the HIF-1 pathway as their exclusive target. However, the fact that some drugs may target multiple pathways could be therapeutically beneficial if the other targets are also involved in cancer progression. On the other hand, pleiotropic inhibition of the pathways involved in normal cellular homeostasis could result in an unacceptable toxicity profile *in vivo*. Therefore, the design of more specific HIF-1 targeting agents is likely to become the focus of future research efforts. It is clear that the success of these studies will critically depend on the availability of sensitive screening methodologies (Fig. 3). Cell culture-based library screens that detect the inhibition of the HRE-induced expression of a reporter have two major limitations: they tend to identify the inhibitors of numerous pathways upstream of HIF-1 and the mechanistic basis of identified compounds is not immediately apparent. Hence, secondary screens are necessary to further characterize 'hit' compounds and *in vitro* screening approaches may become increasingly useful.

The formation of an active HIF-1 transcription factor complex and its binding to HREs appear to be particularly promising targets for the development of specific inhibitors (Fig. 3). The first attempt to disrupt protein interactions in this complex led to the identification of

Table 2 Pharmacological inhibitors of HIF-1

Compound	Molecular target	Stage of development	Reference
HIF-1α synthesis			
LY294002	PI3K	preclinical studies	[23]
wortmannin		preclinical studies	
rapamycin	mTOR	clinically used agent	[51]
CCI-779		phase III	
Rad-001		phase II	[52]
topotecan	topoisomerase I	approved drug	[34,36,59]
103D5R	unknown	preclinical studies	[35]
Folding, stabilization and nuclear translocation			
geldanamycin	Hsp90 chaperone	not in clinical use	[43]
17AAG		phase I, Ib and II	
2ME2	microtubule cytoskeleton	approved drug	[45]
Transactivation			
PX-12	Trx-1	phase I	[41]
pleurotin	Trx-1	preclinical studies	
PX-478	Trx reductase (?)	late stage preclinical development	[43]
chetomin	HIF-1α–p300 interaction	preclinical studies	[53]
polyamides	HIF-1–HRE interaction	not tested	[55]
echinomycin		previous clinical trials	[55]

chetomin. A very recent study tested the potential of HIF-1–HRE and HIF-1α–HIF-1β (G. Mellilo, personal communication) interactions for *in vitro* high-throughput screening. One such screen identified a promising agent, echinomycin, that disrupts the HIF-1–HRE interaction, but not other tested transcription factor–DNA interactions.

Modulation of the physiologic mechanisms of HIF-1 degradation constitutes a conceptually different approach to inhibiting HIF-1. Large chemical libraries could be screened *in vitro* for the agonists of the enzymes involved in HIF-1α degradation (PHD1-3 and ARD1), and transcriptional activity (FIH-1). Small molecule-mediated reactivation of mutant tumor-suppressors such as pVHL, p53 and PTEN can also be exploited for HIF-1 inhibition in certain cancers [58,61].

Finally, the integration of the elements of rational drug discovery is likely to facilitate both cell-based and *in vitro* screening efforts. The detailed structural information has already been obtained for the complexes of HIF-1α with p300 CH1 and pVHL, and the structures of other important HIF-1 complexes are likely to become available in the near future. In combination with the modern docking algorithms, these structures can be used to optimize binding characteristics of existing compounds as well as to predict novel inhibitor molecules in chemical libraries.

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References

1 Kim JA. Targeted therapies for the treatment of cancer. *Am J Surg* 2003; **186**:264–268.
2 Harris AL. Hypoxia – a key regulatory factor in tumour growth. *Nat Rev Cancer* 2002; **2**:38–47.
3 Brat DJ, Castellano-Sanchez AA, Hunter SB, Pecot M, Cohen C, Hammond EH, *et al.* Pseudopalisades in glioblastoma are hypoxic, express extracellular matrix proteases, and are formed by an actively migrating cell population. *Cancer Res* 2004; **64**:920–927.
4 Kaur B, Khwaja FW, Severson EA, Matheny SL, Brat DJ, Van Meir EG. Hypoxia and the hypoxia-inducible-factor pathway in glioma growth and angiogenesis. *Neuro-oncology* 2005; **7**:134–153.
5 Brown JM, Giaccia AJ. The unique physiology of solid tumors: opportunities (and problems) for cancer therapy. *Cancer Res* 1998; **58**: 1408–1416.
6 Zhong H, De Marzo AM, Laughner E, Lim M, Hilton DA, Zagzag D, *et al.* Overexpression of hypoxia-inducible factor 1alpha in common human cancers and their metastases. *Cancer Res* 1999; **59**:5830–5835.
7 Bos R, Zhong H, Hanrahan CF, Mommers EC, Semenza GL, Pinedo HM, *et al.* Levels of hypoxia-inducible factor-1 alpha during breast carcinogenesis. *J Natl Cancer Inst* 2001; **93**:309–314.
8 Zhong H, Semenza GL, Simons JW, De Marzo AM. Up-regulation of hypoxia-inducible factor 1alpha is an early event in prostate carcinogenesis. *Cancer Detect Prev* 2004; **28**:88–93.
9 Birner P, Schindl M, Obermair A, Plank C, Breitenecker G, Oberhuber G. Overexpression of hypoxia-inducible factor 1alpha is a marker for an unfavorable prognosis in early-stage invasive cervical cancer. *Cancer Res* 2000; **60**:4693–4696.
10 Aebersold DM, Burri P, Beer KT, Laissue J, Djonov V, Greiner RH, *et al.* Expression of hypoxia-inducible factor-1alpha: a novel predictive and prognostic parameter in the radiotherapy of oropharyngeal cancer. *Cancer Res* 2001; **61**:2911–2916.
11 Beasley NJ, Leek R, Alam M, Turley H, Cox GJ, Gatter K, *et al.* Hypoxia-inducible factors HIF-1alpha and HIF-2alpha in head and neck cancer: relationship to tumor biology and treatment outcome in surgically resected patients. *Cancer Res* 2002; **62**:2493–2497.
12 Volm M, Koomagi R. Hypoxia-inducible factor (HIF-1) and its relationship to apoptosis and proliferation in lung cancer. *Anticancer Res* 2000; **20**: 1527–1533.
13 Zagzag D, Zhong H, Scalzitti JM, Laughner E, Simons JW, Semenza GL. Expression of hypoxia-inducible factor 1alpha in brain tumors: association with angiogenesis, invasion, and progression. *Cancer* 2000; **88**: 2606–2618.
14 Brat DJ, Van Meir EG. Vaso-occlusive and prothrombotic mechanisms associated with tumor hypoxia, necrosis, and accelerated growth in glioblastoma. *Lab Invest* 2004; **84**:397–405.
15 Zagorska A, Dulak J. HIF-1: the knowns and unknowns of hypoxia sensing. *Acta Biochim Pol* 2004; **51**:563–585.
16 Poellinger L, Johnson RS. HIF-1 and hypoxic response: the plot thickens. *Curr Opin Genet Dev* 2004; **14**:81–85.
17 Bruick RK, McKnight SL. A conserved family of prolyl-4-hydroxylases that modify HIF. *Science* 2001; **294**:1337–1340.

18 Jaakkola P, Mole DR, Tian YM, Wilson MI, Gielbert J, Gaskell SJ, *et al.* Targeting of HIF-alpha to the von Hippel–Lindau ubiquitylation complex by O_2-regulated prolyl hydroxylation. *Science* 2001; **292**:468–472.

19 Jeong JW, Bae MK, Ahn MY, Kim SH, Sohn TK, Bae MH, *et al.* Regulation and destabilization of HIF-1alpha by ARD1-mediated acetylation. *Cell* 2002; **111**:709–720.

20 Mahon PC, Hirota K, Semenza GL. FIH-1: a novel protein that interacts with HIF-1alpha and VHL to mediate repression of HIF-1 transcriptional activity. *Genes Dev* 2001; **15**:2675–2686.

21 Lang KJ, Kappel A, Goodall GJ. Hypoxia-inducible factor-1alpha mRNA contains an internal ribosome entry site that allows efficient translation during normoxia and hypoxia. *Mol Biol Cell* 2002; **13**:1792–1801.

22 Minchenko A, Salceda S, Bauer T, Caro J. Hypoxia regulatory elements of the human vascular endothelial growth factor gene. *Cell Mol Biol Res* 1994; **40**:35–39.

23 Jiang BH, Jiang G, Zheng JZ, Lu Z, Hunter T, Vogt PK. Phosphatidylinositol 3-kinase signaling controls levels of hypoxia-inducible factor 1. *Cell Growth Differ* 2001; **12**:363–369.

24 Li YM, Zhou BP, Deng J, Pan Y, Hay N, Hung MC. A hypoxia-independent hypoxia-inducible factor-1 activation pathway induced by phosphatidylinositol-3 kinase/Akt in HER2 overexpressing cells. *Cancer Res* 2005; **65**:3257–3263.

25 Melillo G. HIF-1: a target for cancer, ischemia and inflammation – too good to be true?. *Cell Cycle* 2004; **3**:154–155.

26 Yeo EJ, Chun YS, Park JW. New anticancer strategies targeting HIF-1. *Biochem Pharmacol* 2004; **68**:1061–1069.

27 Giaccia A, Siim BG, Johnson RS. HIF-1 as a target for drug development. *Nat Rev Drug Discov* 2003; **2**:803–811.

28 Semenza GL. Targeting HIF-1 for cancer therapy. *Nat Rev Cancer* 2003; **3**:721–732.

29 Brown JM, Siim BG. Hypoxia-specific cytotoxins in cancer therapy. *Semin Radiat Oncol* 1996; **6**:22–36.

30 Post DE, Devi NS, Li Z, Brat DJ, Kaur B, Nicholson A, *et al.* Cancer therapy with a replicating oncolytic adenovirus targeting the hypoxic microenvironment of tumors. *Clin Cancer Res* 2004; **10**:8603–8612.

31 Post DE, Van Meir EG. A novel hypoxia-inducible factor (HIF) activated oncolytic adenovirus for cancer therapy. *Oncogene* 2003; **22**: 2065–2072.

32 Kaufman B, Scharf O, Arbeit J, Ashcroft M, Brown JM, Bruick RK, *et al.* Proceedings of the Oxygen Homeostasis/Hypoxia Meeting. *Cancer Res* 2004; **64**:3350–3356.

33 Powis G, Kirkpatrick L. Hypoxia inducible factor-1alpha as a cancer drug target. *Mol Cancer Ther* 2004; **3**:647–654.

34 Rapisarda A, Uranchimeg B, Scudiero DA, Selby M, Sausville EA, Shoemaker RH, *et al.* Identification of small molecule inhibitors of hypoxia-inducible factor 1 transcriptional activation pathway. *Cancer Res* 2002; **62**:4316–4324.

35 Tan C, de Noronha RG, Roecker AJ, Pyrzynska B, Khwaja F, Zhang Z, *et al.* Identification of a novel small-molecule inhibitor of the hypoxia-inducible factor 1 pathway. *Cancer Res* 2005; **65**:605–612.

36 Rapisarda A, Uranchimeg B, Sordet O, Pommier Y, Shoemaker RH, Melillo G. Topoisomerase I-mediated inhibition of hypoxia-inducible factor **1**: mechanism and therapeutic implications. *Cancer Res* 2004; **64**: 1475–1482.

37 Post DE, Van Meir EG. Generation of bidirectional hypoxia/HIF-responsive expression vectors to target gene expression to hypoxic cells. *Gene Ther* 2001; **8**:1801–1807.

38 Komar AA, Hatzoglou M. Internal ribosome entry sites in cellular mRNAs: the mystery of their existence. *J Biol Chem* 2005; in press.

39 Carrero P, Okamoto K, Coumailleau P, O'Brien S, Tanaka H, Poellinger L. Redox-regulated recruitment of the transcriptional coactivators CREB-binding protein and SRC-1 to hypoxia-inducible factor 1alpha. *Mol Cell Biol* 2000; **20**:402–415.

40 Welsh SJ, Bellamy WT, Briehl MM, Powis G. The redox protein thioredoxin-1 (Trx-1) increases hypoxia-inducible factor 1alpha protein expression: Trx-1 overexpression results in increased vascular endothelial growth factor production and enhanced tumor angiogenesis. *Cancer Res* 2002; **62**:5089–5095.

41 Welsh SJ, Williams RR, Birmingham A, Newman DJ, Kirkpatrick DL, Powis G. The thioredoxin redox inhibitors 1-methylpropyl 2-imidazolyl disulfide and pleurotin inhibit hypoxia-induced factor 1alpha and vascular endothelial growth factor formation. *Mol Cancer Ther* 2003; **2**:235–243.

42 Welsh SJ, Powis G. Hypoxia inducible factor as a cancer drug target. *Curr Cancer Drug Targets* 2003; **3**:391–405.

43 Welsh S, Williams R, Kirkpatrick L, Paine-Murrieta G, Powis G. Antitumor activity and pharmacodynamic properties of PX-478, an inhibitor of hypoxia-inducible factor-1alpha. *Mol Cancer Ther* 2004; **3**:233–244.

44 Lakhani NJ, Sarkar MA, Venitz J, Figg WD. 2-Methoxyestradiol, a promising anticancer agent. *Pharmacotherapy* 2003; **23**:165–172.

45 Mabjeesh NJ, Escuin D, LaVallee TM, Pribluda VS, Swartz GM, Johnson MS, *et al.* 2ME2 inhibits tumor growth and angiogenesis by disrupting microtubules and dysregulating HIF. *Cancer Cell* 2003; **3**:363–375.

46 Goetz MP, Toft DO, Ames MM, Erlichman C. The Hsp90 chaperone complex as a novel target for cancer therapy. *Ann Oncol* 2003; **14**:1169–1176.

47 Mabjeesh NJ, Post DE, Willard MT, Kaur B, Van Meir EG, Simons JW, *et al.* Geldanamycin induces degradation of hypoxia-inducible factor 1alpha protein via the proteosome pathway in prostate cancer cells. *Cancer Res* 2002; **62**:2478–2482.

48 Grem JL, Morrison G, Guo XD, Agnew E, Takimoto CH, Thomas R, *et al.* Phase I and pharmacologic study of 17-(allylamino)-17-demethoxygeldanamycin in adult patients with solid tumors. *J Clin Oncol* 2005; **23**:1885–1893.

49 Zundel W, Schindler C, Haas-Kogan D, Koong A, Kaper F, Chen E, *et al.* Loss of PTEN facilitates HIF-1-mediated gene expression. *Genes Dev* 2000; **14**:391–396.

50 Meric-Bernstam F, Mills GB. Mammalian target of rapamycin. *Semin Oncol* 2004; **31**:10–17; discussion 33.

51 Hudson CC, Liu M, Chiang GG, Otterness DM, Loomis DC, Kaper F, *et al.* Regulation of hypoxia-inducible factor 1alpha expression and function by the mammalian target of rapamycin. *Mol Cell Biol* 2002; **22**:7004–7014.

52 Majumder PK, Febbo PG, Bikoff R, Berger R, Xue Q, McMahon LM, *et al.* mTOR inhibition reverses Akt-dependent prostate intraepithelial neoplasia through regulation of apoptotic and HIF-1-dependent pathways. *Nat Med* 2004; **10**:594–601.

53 Kung AL, Zabludoff SD, France DS, Freedman SJ, Tanner EA, Vieira A, *et al.* Small molecule blockade of transcriptional coactivation of the hypoxia-inducible factor pathway. *Cancer Cell* 2004; **6**:33–43.

54 Kung AL, Wang S, Klco JM, Kaelin WG, Livingston DM. Suppression of tumor growth through disruption of hypoxia-inducible transcription. *Nat Med* 2000; **6**:1335–1340.

55 Olenyuk BZ, Zhang GJ, Klco JM, Nickols NG, Kaelin Jr WG, Dervan PB. Inhibition of vascular endothelial growth factor with a sequence-specific hypoxia response element antagonist. *Proc Natl Acad Sci USA* 2004; **101**:16768–16773.

56 Gradishar WJ, Vogelzang NJ, Kilton LJ, Leibach SJ, Rademaker AW, French S, *et al.* A phase II clinical trial of echinomycin in metastatic soft tissue sarcoma. An Illinois Cancer Center Study. *Invest New Drugs* 1995; **13**:171–174.

57 Wadler S, Tenteromano L, Cazenave L, Sparano JA, Greenwald ES, Rozenblit A, *et al.* Phase II trial of echinomycin in patients with advanced or recurrent colorectal cancer. *Cancer Chemother Pharmacol* 1994; **34**: 266–269.

58 North S, Pluquet O, Maurici D, El-Ghissassi F, Hainaut P. Restoration of wild-type conformation and activity of a temperature-sensitive mutant of p53 (p53(V2T2M) by the cytoprotective aminothiol WR1065 in the esophageal cancer cell line TE-1. *Mol Carcinog* 2002; **33**:181–188.

59 Rapisarda A, Zalek J, Hollingshead M, Braunschweig T, Uranchimeg B, Bonomi CA, *et al.* Schedule-dependent inhibition of hypoxia-inducible factor-1alpha protein accumulation, angiogenesis, and tumor growth by topotecan in U251-HRE glioblastoma xenografts. *Cancer Res* 2004; **64**:6845–6848.

60 Kong D, Park EJ, Stephen AG, Calvani M, Cardellina JH, Monks A, *et al.* Echinomycin, a small molecule inhibitor of HIF-1 DNA binding activity. *Cancer Res* 2005; in press.

61 Issaeva N, Bozko P, Enge M, Protopopova M, Verhoef LG, Masucci M, *et al.* Small molecule RITA binds to p53, blocks p53-HDM-2 interaction and activates p53 function in tumors. *Nat Med* 2004; **10**:1321–1328.