HIF-1 AS A TARGET FOR DRUG DEVELOPMENT

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Sensing and responding to fluxes in oxygen tension is perhaps the single most important variable in physiology, and animal tissues have developed a number of essential mechanisms to cope with the stress of low physiological oxygen levels, or hypoxia. Among these coping mechanisms is the response mediated by the hypoxia-inducible transcription factor, or HIF-1. HIF-1 is an essential component in changing the transcriptional repertoire of tissues as oxygen levels drop, and could prove to be a very important target for drug development, as treatments evolve for diseases, such as cancer, heart disease and stroke, in which hypoxia is a central aspect.

HYPOXIA-RESPONSE ELEMENT (HRE). Initially identified as a 50-base-pair sequence in the 3′ flanking region of the erythropoietin gene. Although the core DNA binding element is 5′-ACGTG-3′, flanking sequences are also important in HRE functionality. Approximately 30 genes have been found to possess HREs.

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Adaptation of cells to an anaerobic environment is achieved by the transcriptional induction of genes that are involved in glycolysis[1], haematopoiesis[2,3], angiogenesis[4], invasion[5] and regulation of vascular tone[6], as well as a number of others. Perhaps the earliest insight into the transcriptional regulation of gene expression by hypoxia came from studies on erythropoietin (*EPO*) gene regulation. In the early 1990s, several groups demonstrated that the hypoxic inducibility of the *EPO* gene is due in part to a HYPOXIA-RESPONSE ELEMENT (HRE), 5'-ACGTG-3', localized in its 3' flanking region[7,8]. The transcription factor that bound this HRE was designated the hypoxia-inducible factor-1 (HIF-1). HIF-1 is composed of two subunits: an oxygen-sensitive HIF-1α subunit, and a constitutively expressed HIF-1β subunit (also known as ARNT, the aryl hydrocarbon receptor nuclear translocator)[9]. Both HIF-1α and HIF-1β are members of the basic helix–loop–helix PER/ARNT/SIM (HLH–PAS) family of transcription factors[10]. In contrast to the constitutively expressed HIF-1β subunit, HIF-1α is an oxygen-labile protein that becomes stabilized in response to hypoxia, iron chelators and divalent cations. Under hypoxic conditions in cell culture, HIF-1α messenger RNA levels do not change, but HIF-1α protein levels increase[11,12]. Interestingly, the ability to confer oxygen sensitivity to heterologous proteins is transferable from HIF-1α to other proteins[13,14]. In fact, fusion of different HIF-1α domains to the yeast GAL4 DNA-BINDING PROTEIN has identified two separable hypoxia-responsive domains. One domain is localized between residues 531–575, and is important in modulating HIF-1α protein stability, and is therefore referred to as the OXYGEN-DEPENDENT DEGRADATION (ODD) domain[13,15]. The second domain is localized between residues 786–826 (referred to as C-transactivating domain (C-TAD)), and is involved in modulating transcriptional activation of HIF-1α under hypoxic conditions. The finding that transfer of residues 531–575 to a heterologous protein can confer oxygen sensitivity indicates that the oxygen sensor itself does not have to be directly associated with HIF-1. Interestingly, two additional members of the HIF-1α family, designated HIF-2α (also known as endothelial PAS domain protein 1 (EPAS1), or MOP2)[16–19] and HIF-3α, have been identified[20]. HIF-2α is highly similar to HIF-1α in both structure and function, but exhibits more restricted tissue-specific expression, and might also be differentially regulated by nuclear translocation. HIF-3α also exhibits conservation with HIF-1α and HIF-2α in the HLH and PAS domains, but does not possess a hypoxia-inducible domain[20].

Role of VHL in regulation of HIF-1α

Hypoxia regulates HIF-1 at the level of protein stability by inhibiting its UBIQUITIN-MEDIATED DEGRADATION[12,21]. This concept is supported by studies on cell lines derived from tumours that have lost the VON HIPPEL-LINDAU (*VHL*) tumour-suppressor gene. These VHL-deficient cells exhibit aerobic HIF-1α protein expression[22,23]. Tumours

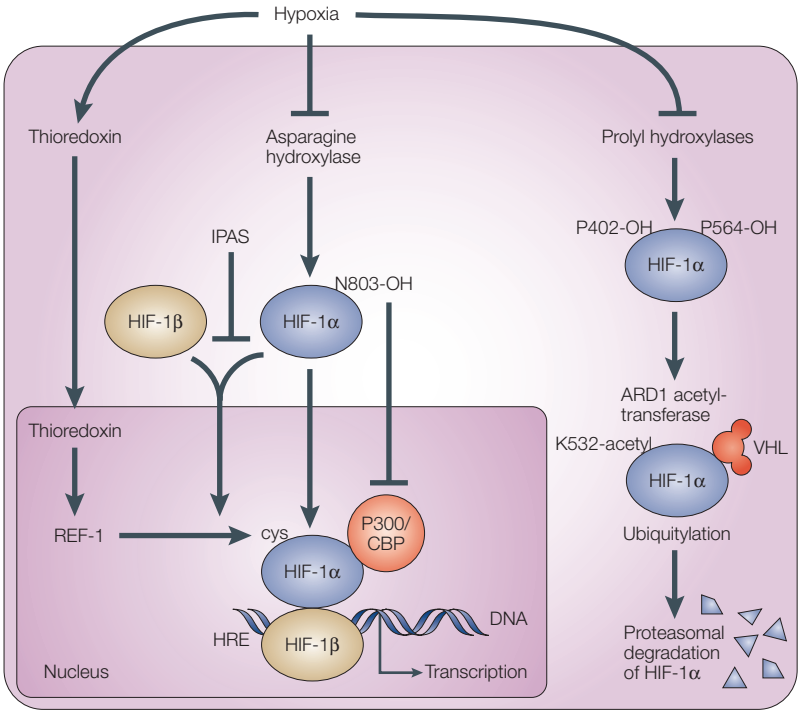

Figure 1 | **Mechanisms of HIF-1α regulation under aerobic and hypoxic conditions.** Under aerobic conditions, HIF-1α is hydroxylated on proline 402 and proline 564. The proline hydroxylations are necessary for binding to von Hippel-Lindau (VHL) and ubiquitin-mediated degradation by the proteasome. The asparagine hydroxylation prevents binding to p300/CBP. A splice derivative of HIF-3α called inhibitory PAS (IPAS), as it only possesses the PAS domain, competes for HIF-1β binding. Maintenance of cysteine in a reduced state in the transactivation domain (TAD) is essential for p300/CBP binding. Compounds that inhibit thioredoxin inhibit HIF-1α-mediated transactivation. HIF-1α and HIF-1β both translocate to the nucleus to transactivate genes such as vascular endothelial growth factor (VEGF) that possess hypoxia-response elements (HREs). PAS, PER/ARNT/SIM; REF-1, redox factor 1.

such as renal cell carcinomas that possess mutations in *VHL* also exhibit high aerobic expression of HIF-1-regulated genes, whereas re-introduction of wild-type VHL substantially reduces the aerobic level of HIF-1α protein, to that found in untransformed or transformed cells that express wild-type VHL. In addition, protein stability of HIF-1α is easily separable from its heterodimerization with HIF-1β, as cells that are HIF-1β-deficient still exhibit HIF-1α stabilization[24].

The functional relationship between VHL and the ubiquitylation system arises because the VHL protein forms a complex with transcription elongation factors (TCEs) B1 and B2 (also known as elongins C and B, respectively), the cullin 2 (CUL2) protein, and the RING-H2 protein ring-box 1 (RBX1)[25–29]. Significant homology exists between TCEB2 and ubiquitin itself, whereas elongin C, CUL2 and RBX1 are closely related to the components of the SCF (S-kinase-associated protein 1 (SKP1)/CUL1/F-box) family of E3 ubiquitin–ligase complexes. These are directly involved in the ubiquitylation of proteins targeted for degradation by the 26S proteasome[30–32]. The VHL protein folds into two functional domains, α and β. The α-domain of VHL binds to elongin C through amino acids 157–171, and their interaction is stabilized in the presence of TCEB2[23,33,34]. Frequent natural mutations within amino acids 157–171 underline the importance of the interaction between VHL and elongin C. By contrast, the β-domain of VHL directly interacts with HIF-1α[23,35,36]. The β-domain is also subjected to many natural mutations, and tyrosine 98 is the second-most frequently mutated residue in VHL. This and other missense mutations in the β-domain often result in diminished interaction with HIF-1α[23].

Deletion analysis indicates that the interaction of VHL with HIF-1α requires amino acids 530–650 of HIF-1α, which in turn overlap with the previously identified ODD[23]. Located in this domain is a crucially important proline residue at position 564, which becomes hydroxylated by a family of iron (II)-dependent PROLYL HYDROXYLASES; these use molecular oxygen as a substrate. These prolyl hydroxylases directly sense the availability of oxygen[37–40]. Hydroxylation of proline 564 is necessary and sufficient for the interaction of HIF-1α with VHL (FIG. 1).

Although not involved in the stabilization of HIF-1α, the C-TAD is involved in modulating transcriptional activation of HIF-1α under hypoxic conditions. Under these conditions, C-TAD is able to interact with transcriptional co-activators such as P300/CBP[41–43]. However, this interaction requires the inhibition of another oxygen-dependent hydroxylation event; that is, the hydroxylation of the asparagine residue in the conserved domain YDCEVNV/AP, located within C-TAD[44] (FIG. 1). Recently, the gene that is responsible for the hydroxylation of asparagine has been identified as encoding the HIF-1α inhibitor (HIF-1αN, also known as factor inhibiting HIF-1 (FIH))[45–48]. An additional mechanism of regulating HIF-1 transactivation potential is through thioredoxin. HIF-1α is regulated by redox factor 1 (REF-1) and thioredoxin[41,49–51] (FIG. 1). Taken together, these observations demonstrate that HIF-1 activity, and eventually the cellular response to hypoxia, are regulated at multiple discrete levels.

It is certainly of great interest to identify any natural mutations in the hydroxylases that modify HIF-1α, at either the proline or the asparagine residues. So far, three prolyl hydroxylases have been identified, two of which are hypoxia-inducible[37,40]. By contrast, only one ASPARAGINE HYDROXYLASE (FIH) has been identified[45–48]. A recent study by Jeong *et al.* showed that ACETYLATION of HIF-1α by a yeast acetyltransferase homologue ARD1 is crucial for HIF-1α degradation, although by an as-yet-unknown mechanism[52]. Another mechanism of regulating HIF-1 is the inhibition of HIF-1α and HIF-1β dimerization, which is achieved through competitive binding of an inhibitory PAS (IPAS) molecule[53,54] (FIG.1). This protein is able to dimerize with HIF-1α molecules, but lacks a transactivation domain. Recently, IPAS has been reported to be generated by alternative splicing of HIF-3α. Alternatively spliced variants of HIF-1α and HIF-1β have also been reported, indicating that these molecules could represent an additional mechanism of maintaining stringent HIF-1 regulation[55–57].

HLH–PAS DOMAIN
HLH is a helix–loop–helix motif that facilitates dimerization and DNA binding and is found in a substantial number of transcription factors. The PAS domain was named after PER, ARNT and SIM proteins that represent the first proteins in which this motif was identified. Functionally, the PAS domain facilitates protein–protein interactions between family members.

GAL4 DNA-BINDING PROTEIN
A transcriptional activator identified in yeast, which, because it is specific for yeast, is used to make fusion proteins to study mammalian transcriptional regulators.

For HIF-1 to be functional as a transcription factor, it must translocate to the nucleus. HIF-1 translocation could also serve as a target for inhibiting HIF-1[116]. Hydroxylation of HIF-1α, and the formation of complexes with VHL, represent another unique mechanism to regulate protein stability that could possibly be pharmacologically manipulated. The requirement for HIF-1α-binding proteins, dimerization and nuclear translocation for activity also provides additional possible targets for HIF-1α inhibitors.

Targeting HIF-1

Given the central role that HIF-1-driven transcription factor activity has in compensating for loss of oxygen, it is clear that modulation of that activity could be a potent mechanism for treating a wide range of hypoxia-related pathologies. Increases in HIF-1 activity could increase survival during hypoxia, and increase angiogenesis at sites of vascular disruption or dysfunction. Decreased HIF-1 activity could prevent the survival or angiogenic activity of pathological tissues with hypoxic regions; that is, solid tumours. Recently, evidence has accumulated that inhibition of HIF-1 activity could also act to prevent inflammation, by virtue of its essential role in the activation and infiltration of macrophages and neutrophils into affected tissues. All of these activities make the HIF-1 transcription factor an attractive nexus for drug development, as is evident by the agents already characterized in TABLE 1.

Is HIF-1α a good target for cancer therapy? Untransformed mammalian cells have evolved an intricate series of mechanisms to tightly regulate HIF-1α activity under aerobic conditions (FIG. 1). However, tumour cells might have a greater requirement for HIF-1, and many seem to express HIF-1α both under hypoxic and oxic conditions[58]. In tumours, HIF-1α is overexpressed compared with adjoining normal tissue. This overexpression of HIF-1α is due to both hypoxia-dependent as well as hypoxia-independent pathways, such as oncogene activation and glucose deprivation[59–62]. Most importantly, overexpression of HIF-1α or HIF-2α have been implicated as poor prognostic indicators for a variety of tumours[63]. A question that often arises is whether HIF-1α is simply a surrogate marker for tumour response, reflecting tumour hypoxia, or whether it plays an active role in tumour growth and invasion. Studies by Ryan *et al.* were the first to demonstrate that loss of HIF-1 severely impedes tumour growth and the induction of pro-angiogenic gene expression[64]. Although the role of HIF-1α in human tumours remains correlative, in transplanted tumours in immunodeficient mice, loss of HIF-1α or HIF-1β results in reduced tumour growth, decreased angiogenesis and increased responsiveness to radiotherapy[64–67]. These studies demonstrate that loss of either HIF-1 subunit leads to decreased tumour growth. However, one study proposed that loss of HIF-1α leads to enhanced tumour growth[66]. Although this latter study indicates that, in some cases, inhibiting HIF-1α promotes tumour growth, the conclusion of the study is somewhat questionable as the tumour derived from the wild-type cells grew in an unusually slow manner.

Targeting HIF-1α to inhibit tumour growth? The strategy to identify small-molecule inhibitors of HIF-1α or its target genes is supported by published genetic and pharmacological studies. Different approaches have been used to inhibit HIF-1α gene transcription: through antisense strategies, through inhibition of the ability of HIF-1α to interact with proteins that modulate its activity, or through inhibition of signal transduction pathways. The use of antisense HIF-1α is experimentally relevant in cell culture, but would be difficult to use clinically with current technology[68]. However, two interacting HIF-1α proteins, heat-shock protein 90 (HSP90) and thioredoxin, can be pharmacologically targeted to inhibit HIF-1. HSP90 is a molecular chaperone involved in properly folding HIF-1α protein[69,70]. Previous studies with HSP90 inhibitors, such as geldanamycin and KF58333, indicate that HIF-1α stabilization can be decreased when HSP90 binding is inhibited[71,72]. At present, geldanamycin has been derivatized to a less toxic form that could potentially be used in the clinic to inhibit HIF-1 (REFS 73,74). It remains to be determined whether the antitumour effect of these agents is due to inhibition of HIF-1α, or other targets that also require HSP90 for proper protein processing.

Recent studies have indicated that inhibition of thioredoxin-1 (TRX1), an ubiquitously expressed redox protein, results in inhibition of HIF-1α, and its transcriptional target, the angiogenic factor vascular endothelial growth factor (VEGF), in transformed cells. Inhibition of thioredoxin is in some ways an attractive goal, as there are several potent inhibitors of this enzyme: PX-12, a thioalkylator of cysteine 73 of TRX-1, and pleurotin, an irreversible inhibitor of TRX-1 (REF. 75). PX-12 is presently in Phase I clinical trials, and has shown antitumour activity in transplanted tumours in immunodeficient mice.

Previous studies have shown that the oncogenes *RAS*[71,72,76] and *SRC* are able to activate HIF-1 (REFS 59,77,78). Pharmacological agents that are directed at the activity of the *RAS* oncogene, such as farnesyl transferase inhibitors, could potentially exert their antitumour effects through HIF-1α, as tumours treated with these inhibitors no longer possess hypoxic regions[79]. Src kinase inhibitors have also been developed, but their effect on HIF-1α has not been evaluated.

Tumour-suppressor genes encoding proteins such as VHL, phosphatase and tensin homologue (PTEN) and p53 have all been reported to inhibit HIF-1α function, but in different ways. The *VHL* tumour-suppressor gene is a ubiquitously expressed cellular regulator of HIF-1α half-life. As mentioned above, VHL is an E3 ligase that binds HIF-1α, and targets it for ubiquitin-mediated degradation[80]. Tumour cells that have decreased VHL expression have increased concentrations of HIF-1α, as well as of HIF-1α target gene products, under aerobic conditions[80].

In contrast to VHL, the *PTEN* tumour-suppressor gene inhibits HIF-1α stabilization and HIF-1 target genes by antagonizing the phosphatidylinositol 3-kinase (PI3K) pathway. PTEN is a lipid phosphatase that

OXYGEN-DEPENDENT DEGRADATION (ODD). The ODD domain of HIF-1α binds to VHL under aerobic conditions. Deletion of this domain results in a HIF-1α protein that is oxygen insensitive and constitutively expressed under aerobic conditions.

UBIQUITIN-MEDIATED DEGRADATION
The energy-requiring process of covalently linking ubiquitin to lysine residues of a substrate protein to signal protein degradation.

VON HIPPEL-LINDAU (VHL). A tumour-suppressor gene that possesses two substrate-binding domains, -α and -β. The α-domain binds to elongin C and CUL2, proteins that possess sequence similarity with proteins known to be involved in ubiquitin-mediated degradation. The β-domain of VHL binds HIF-1α.

PROLYL HYDROXYLATION
A protein modification mediated by an evolutionarily conserved group of iron-dependent enzymes termed prolyl hydroxylases (PHDs). As they require oxygen for their activity, they have been implicated as the oxygen sensor that regulates HIF-1α stabilization. Loss of PHD activity in *Caenorhabditis elegans* and, more recently, in mammalian cells, has resulted in stabilization of HIF-1α under aerobic conditions.

ASPARAGINE HYDROXYLATION
This modification of HIF-1α on asparagine 803 has been implicated in the control of HIF-1 transactivation potential. The gene identified that controls this modification is termed *FIH*.

ACETYLATION
Acetylation has previously been implicated in promoting transcriptional activation. By contrast, acetylation of HIF-1α on lysine 532 by ARD1 has been shown to be involved in its degradation by the proteasome.

Table 1 | **HIF-1/HIF-2 pharmacological inhibitors**

Inhibitor	Target	References
YC-1 stimulator (sGC)	Soluble guanylyl cycle	106
2-ME2	Microtubule destabilizer	103
Taxol	Microtubule stabilizer	103
Vincristine	Microtubule stabilizer	103
1-Methylpropyl-2-imidazolyl disulphide	Thioredoxin reductase	75
Pleurotin	Thioredoxin reductase	75
Rapamycin/CCI779	TOR	90,95,96
LY294002, wortmannin	PI3K	71,82,83,84,86,96
Geldanamycin	HSP90	70,73
Quinocarmycin	HRE transcriptional activity	99
Topotecan	Topoisomerase I	99
PD98059	MEKK	91,92

2-ME, 2-methyoxyoestradiol; HIF, hypoxia-inducible factor; HRE, hypoxia-response element; HSP90, heat-shock protein 90; MEKK, mitogen-activated protein kinase kinase kinase; PI3K, phosphatidylinositol 3-kinase; TOR, target of rapamycin.

dephosphorylates lipid moieties that are phosphorylated by PI3K. HIF-1 transcription activity and the expression of angiogenic factors are subjected to regulation by the PI3K/AKT pathway[81–84]. Therefore, the tumour-suppressor gene *PTEN* could also serve to check the hypoxia-induced stimulation of the PI3K–HIF–VEGF pathway. The *PTEN* tumour-suppressor gene is mutated or inactivated in a large percentage of glioblastomas[85]; these possess median pO_2 well below those found in other solid tumours. Using *PTEN*-deficient glioblastoma cell lines, it was shown that PTEN inhibits HIF-1 activation and VEGF induction by hypoxia to the same extent as wortmannin, a potent PI3K inhibitor[84]. Interestingly, PTEN inhibited the hypoxia-stimulated accumulation of HIF-1α in glioblastoma-derived cell lines, although not through direct phosphorylation of, or interaction with, the protein. Similar observations on the role of PI3K/AKT have been made in other tumour cells, such as human breast[62,71] and prostate cancer cells[82]. It is noteworthy that the PI3K/AKT pathway can regulate HIF-1α by increasing its translation through a mechanism that is independent of that which controls its stabilization[62,77]. These observations indicate that tumour cells deficient in *PTEN* tend to have elevated HIF-1α activity and therefore increased production of pro-angiogenic factors under aerobic conditions. However, a recent study[86] indicates that the effect of the PI3K/AKT pathway on hypoxia-induced activation of HIF-1α might be cell-type specific. In some cell types (HepG2 and HEK293T), no correlation was found between the activation of the PI3K/AKT pathway and HIF-1α activity under hypoxia. These findings indicate that multiple, and perhaps redundant, regulatory mechanisms exist that modulate HIF-1α in response to oncogenic stimuli and hypoxia in different cell types (FIG. 2).

Another mechanism that is implicated in the negative regulation of HIF-1α is binding of the tumour suppressor p53 (REFS 87,88). However, the role of p53 in regulating HIF-1α activity is still controversial. It is unclear how physiologically significant p53 and HIF-1α interactions are, as tumours that have wild-type p53 still have a robust HIF-1 response to hypoxia. In regards to clinical applicability, gene therapy approaches to introduce VHL, PTEN or p53 into tumours would only affect the regulation of HIF-1α in a small number of tumour cells, without any bystander effects. Therefore, these genes do not represent practical targets for HIF-1α therapy.

Signal transduction pathways could also be targeted to inhibit HIF-1α, although the mechanisms by which they affect HIF-1α expression or downstream effector genes have not been fully elucidated. Two major signalling pathways have been implicated in HIF-1α activation. One pathway, as described above, is mediated through the PI3K lipid signalling pathway. Genetic and pharmacological inhibitors of PI3K result in the inhibition of HIF-1α stabilization or activation, and HIF-1α downstream effector gene expression[71,82–84]. In the PI3K pathway, the AKT kinase has also been proposed to stimulate HIF-1α stabilization and activation, as well as HIF-1α downstream transcriptional targets. Recent studies have indicated that AKT does not directly phosphorylate HIF-1α or bind to it[84]. One potential substrate of AKT that could be the intermediate kinase between AKT and HIF-1α is the translation regulatory protein target of rapamycin (TOR) (also known as RAR-related orphan receptor C (RORC))[89]. A recent study indicates that inhibition of TOR using rapamycin results in decreased HIF-1α stabilization and transactivation[90]. By contrast, overexpression of TOR increases HIF-1α stabilization and transactivation, under both aerobic and hypoxic conditions. Interestingly, *in vitro*, HIF-1α can also be a substrate for TOR kinase. The exact residue that TOR phosphorylates is as yet unknown.

A second signal transduction pathway that has been implicated in HIF-1α activation is the mitogen-activated protein (MAP) kinase pathway[91]. In some cell types, inhibition of MAP kinase activity can prevent HIF-1α activation. However, this pathway might be more involved in the regulation of HIF-1α under aerobic conditions than under hypoxic conditions. Both the PI3K and MAP kinase pathways support a role for growth-factor-receptor signalling in HIF-1α stabilization[92]. Indeed, cells that possess HER2/*neu* mutations exhibit increased amounts of HIF-1α protein stabilization and transcriptional activity[66]. Other growth factors have also been reported to activate HIF-1α[93,94]. The intriguing aspect of inhibiting signal transduction pathways as a means of inhibiting HIF-1α is the existence of pharmacological inhibitors, in particular for the TOR kinase. Rapamycin, a highly specific inhibitor of TOR, is also a potent inhibitor of HIF-1 (REFS 90,95,96). Interestingly, cells that possess PTEN mutations are far more sensitive to rapamycin than cells that possess functional PTEN[97]. As cells that have lost PTEN also have deregulated HIF-1 (REF. 84), the sensitivity of tumours derived from these cells to rapamycin could be in part due to altered concentrations of HIF-1α (FIG. 2). An analogue of rapamycin, CCI779, is presently in clinical trials, and has been suggested to work as an antitumour agent by

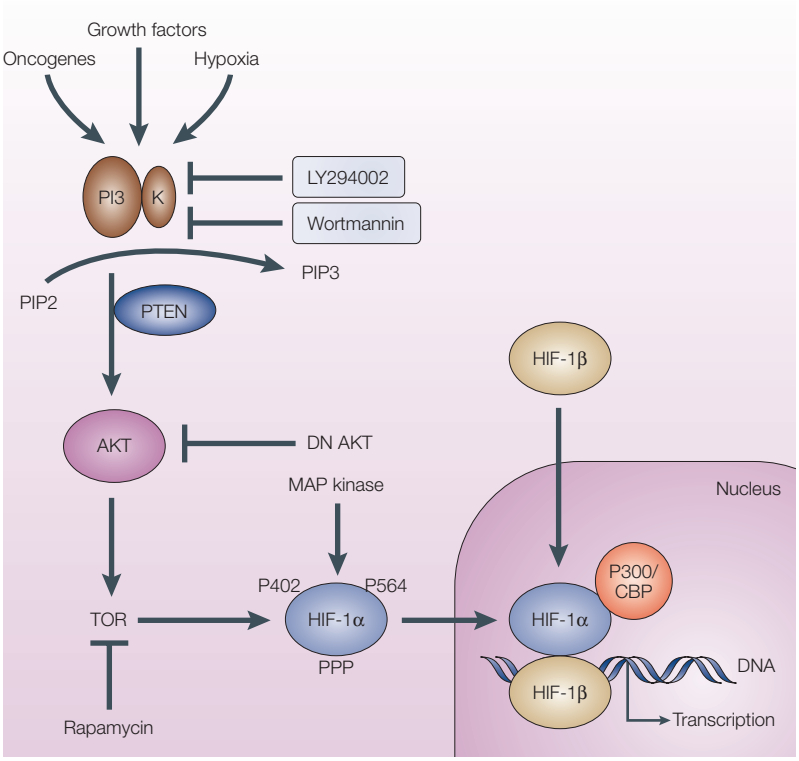

Figure 2 | **Signal transduction pathways implicated in HIF-1α regulation under aerobic and hypoxic conditions.** Oncogenes, growth factors and hypoxia have been documented to stabilize hypoxia-inducible factor (HIF)-1α protein and increase its transactivation potential. Two main pathways have been implicated in this regulation: the lipid signalling pathway PI3K and the mitogen-activated protein (MAP) kinase pathway. The PI3K pathway is thought to increase HIF-1α stabilization through target of rapamycin (TOR). MAP kinase can directly phosphorylate HIF-1α and increase its stabilization. Oncogenic stimuli and growth factors inhibit prolyl hydroxylase activity. DN, dominant negative; PI3K, phosphatidylinositol 3-kinase; PIP, phosphatidylinositol-4,5-biphosphate; PTEN, phosphatase and tensin homologue.

inhibiting expression of pro-angiogenic factors such as VEGF[98]. Whether the antitumour effect of rapamycin is in part due to altered activity of HIF-1α has not yet been directly investigated, but it is highly consistent with past experimental findings. Still, it should be kept in mind that inhibition of TOR by rapamycin will result in many additional protein changes, other than that seen in HIF-1α, due to the function of TOR in translation.

An elegant screen by Rapisarda *et al.* of 2,000 compounds that represent a 'Diversity Set' of the US National Cancer Institute's repository, found two types of HIF inhibitors[99]. One inhibitor, NSC-607097 (DX-52-1), inhibited HIF-1 transcriptional activity, as assayed by an HRE-driven luciferase assay. This compound is an analogue of quinocarmycin, which had antitumour activity against transplanted melanoma cell lines in mice[100], but an undefined toxicity[101]. The second group of compounds found in this study that had activity against HIF-1α were topoisomerase I inhibitors. This group is exemplified by the compound NSC-609699 (topotecan)[101]. This second group of compounds seemed to inhibit both HIF-1 transcriptional activity and HIF-1α stabilization, by as yet unknown mechanisms[99]. Although this screen was instrumental in demonstrating that pharmacological inhibitors of HIF-1α could be screened in a high-throughput fashion, it attempted to exclude compounds that were cytotoxic, on the basis of the changes in expression of a second constitutively expressed luciferase reporter gene.

Recently, two interesting compounds have been identified that inhibit HIF-1α protein stability and transactivation through unique mechanisms. The compound 2-methyoxyoestradiol (2-ME2) is a naturally occurring derivative of oestradiol that is orally active and has been shown to possess anti-angiogenic activity[102]. A recent study by Mabjeesh *et al.* demonstrated that 2-ME2 inhibits HIF-1α at the post-transcriptional level, prevents HIF-1 target gene expression in tumour cells and inhibits HIF-2α in human endothelial cells[103]. The inhibition of HIF-1 and HIF-2 by 2-ME2 is thought be the result of microtubule depolymerization, indicating the importance of the cytoskeleton in HIF regulation. In addition, this same study demonstrated that commonly used chemotherapeutic agents, such as taxol and vincristine, that alter microtubule stability also result in HIF-1 inhibition, indicating that the antitumour effects of these agents might in part result from HIF inhibition. However, a different study reported that the microtubule-stabilizing agents taxol and vinblastine induce HIF-1 stabilization under aerobic conditions; clearly, further studies will be required to understand how microtubule-stabilizing agents affect HIF-1 stability and activity[104].

A second compound that has also been found to have potent antitumour activity is YC-1, a soluble guanylyl-cyclase stimulator[105]. Soluble guanylyl cyclase is a receptor for nitric oxide, a molecule that acts in a pleiotropic manner in regulating angiogenesis and haematopoiesis. Studies by Yeo *et al.* indicate that YC-1 inhibits HIF-1α protein stability and transactivation of downstream effector genes in cell culture studies[106]. Treatment of tumours with YC-1 results in a delay in tumour growth and loss of HIF-1α protein, indicating the inhibition of HIF-1α in tumours as a target of YC-1. However, it is unclear to what extent the antitumour and anti-angiogenic activities of 2-ME2 and YC-1 are dependent on HIF, and to what extent other as yet unidentified targets are also responsible for these antitumour effects. Future studies using HIF-deficient tumour-derived cell lines, as well as VHL-deficient cell lines, will be useful in determining the contributions of HIF inhibition in the antitumour effects of these compounds.

In addition, it is noteworthy that many of the post-translational modifications that are involved in HIF-1α regulation probably represent a dynamic situation between forward and reverse enzymatic reactions. For example, whereas the hydroxylation of HIF-1α by prolyl and asparagine hydroxylases results in decreased HIF-1 stability and transactivation, increased DEHYDROXYLASE activity could stabilize HIF-1. The identity of such a dehydroxylase is still speculative. Less speculative is the existence of approximately one hundred DEUBIQUITYLATION enzymes in the mammalian genome. As the protein half-life of HIF-1α under aerobic and hypoxic conditions is

DEHYDROXYLASE
An enzyme that can remove the hydroxyl group from proline 564 and promote HIF-1α stabilization.

DEUBIQUITINASE
An enzyme that promotes the removal of ubiquitin from a substrate protein such as HIF-1α through the cleavage of isopeptide bonds. The enzymatic activity of a HIF-1α deubiquitinase should increase HIF-1α stabilization.

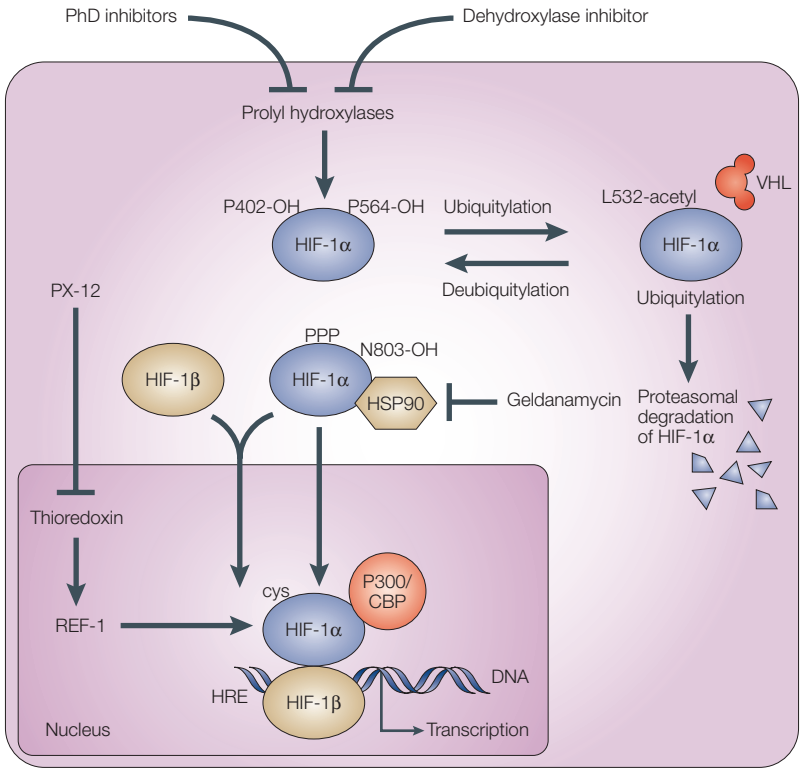

Figure 3 | **Examples of enzymatic steps that have been or could be targeted to inhibit HIF-1α stability or HIF-1 activity.** Current approaches to inhibiting HIF-1α include preventing hypoxia-inducible factor (HIF)-1α from interacting with proteins that modulate its activity, or inhibiting signal transduction pathways. For example, heat-shock protein 90 (HSP90) is a molecular chaperone involved in properly folding HIF-1α protein, and is inhibited by geldanamycin. Recent studies have indicated that inhibition of thioredoxin-1 results in inhibition of HIF-1α. At present, several potent inhibitors of thioredoxin exist, such as PX-12. The protein half-life of HIF-1α is highly dependent on ubiquitylation. The inhibition of the activity of a HIF-1α deubiquitylation enzyme that controls the removal of ubiquitin would be another new approach to inhibiting HIF-1. In contrast to inhibitory activity, increased dehydroxylase activity could result in HIF-1α stabilization and increase its transactivation activity. The identity of such a hydroxylase is still speculative. HRE, hypoxia-response element; PhD, prolyl hydroxylase; REF-1, redox factor-1; VHL, von Hippel-Lindau.

highly dependent on ubiquitylation, the inhibition of the activity of a HIF deubiquitylation enzyme that controls the removal of ubiquitin, especially under hypoxic conditions, would be another new approach to inhibiting HIF-1. The precedence for such a paradigm has recently been reported for the p53 tumour-suppressor gene through the identification of HAUSP, a herpes virus ubiquitin-specific protease[107]. Additionally, when the functional activities of HIF-1α phosphorylation are understood, modulation of phosphatase activity could be a third new approach to modulating HIF activity. All three enzymatic processes represent highly attractive targets that would be amenable to high-throughput screening approaches (FIG. 3).

The advantage of targeting HIF-1

Targeting HIF-1α to selectively kill or inhibit hypoxic tumour cells has now become feasible, on the basis of our understanding of the complex regulation of this protein. In fact, some of the antitumour agents that are now in clinical trials, such as farnesyl transferase inhibitors, PI3K inhibitors and TOR kinase inhibitors, might act in part through inhibiting HIF-1α. The consequences of inhibiting HIF-1 should be multiple. Growth of a multi-cell tumour mass requires angiogenesis[108]. Inhibition of HIF-1 will lead to inhibition of tumour expansion by decreasing pro-angiogenic gene expression[64]. In addition to inhibiting the growth of hypoxic tumour cells, inhibition of HIF-1 could inhibit the growth of tumour cells that possess oncogenic alterations in Ras, Src or Her2/Neu, or tumour cells that have lost the tumour-suppressor genes *VHL* and *PTEN*. Although the effect of loss of HIF-1α in tumours leads to inhibition of tumour growth, it probably does not eliminate tumours.

The modelling and development of potent HYPOXIA-SPECIFIC CYTOTOXINS indicates that the addition of such an agent to radiation can markedly potentiate cell killing[109]. Most importantly, this killing can be considerably greater than if all of the cells in the tumour were fully oxygenated. The basic premise, that hypoxia could aid the use of hypoxic cell cytotoxins, is based on both the dynamic and static ways tumour hypoxia can occur by transient opening and closing of blood vessels, and by metabolic consumption through successive cell layers surrounding a vessel, respectively[109]. Therefore, transient opening and closing of blood vessels and re-hypoxiation after a fraction of radiotherapy can explain the pathophysiological changes in the tumour micro-environment that would provide the necessary micro-environment for HIF-1-dependent cytotoxins to be an advantage when combined with radiotherapy. The advantage of targeting HIF-1 is its rapid response to changes in oxygenation, making it a good target for both transient (perfusion-limited) and chronic (diffusion-limited) hypoxic cells.

FIGURE 4 is a diagrammatic representation of the potential targets for HIF-1-dependent therapeutics. HIF-1-dependent cytotoxics will fall into at least four principal groups. The rapid inhibition of HIF-1 *in vitro* can have multiple effects, depending on the cell type. A tumour cell with elevated levels of HIF-1α is more sensitive to the loss of HIF-1α than a tumour cell with lower levels of HIF-1α (for example, drug A). The reasons for this could be several. First, tumour cells become adapted to elevated levels of HIF-1α, as exemplified by VHL-deficient cells. Sudden changes in the level of HIF-1α could lead to cell death. Second, a compound could induce cell death only when HIF-1 is elevated (drug B). This is similar to the concept of synthetic lethality in yeast: when either one of two genes is altered in expression, yeast are viable; when both genes are altered in expression simultaneously, yeast are not viable. By analogy, a drug could inhibit an essential function (for example, ribosomal function) that would not cause lethality unless HIF-1 is elevated. Third, the compound could inhibit a specific HIF-1 target gene involved in glycolysis that is necessary for the survival of cells that have become adapted to elevated levels of HIF-1 (drug C). Fourth, the increased cytotoxicity of a compound could be the result of its metabolic activation (drug D). For example, tumours express high

HYPOXIA-SPECIFIC CYTOTOXIN
A molecule whose cytotoxic activity is inhibited under aerobic conditions and increased under hypoxic conditions.

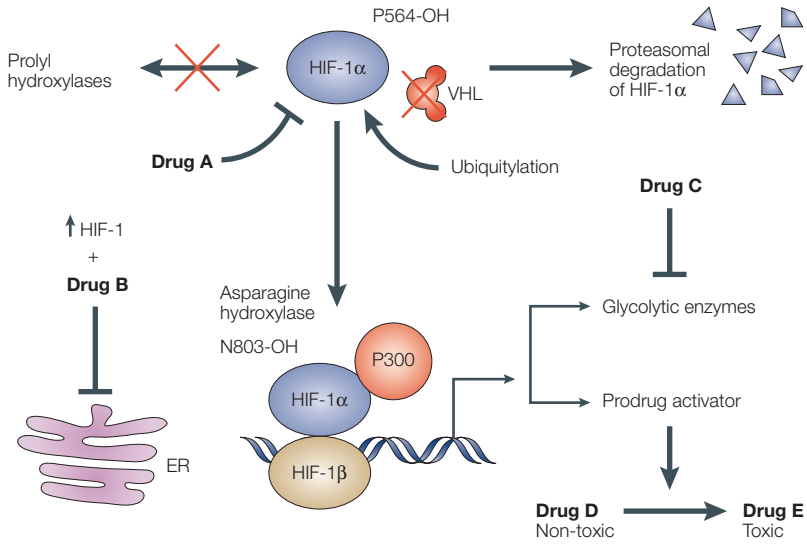

Figure 4 | **Cartoon depicting potential intracellular targets that could result in HIF-1-specific cytotoxicity.** A tumour cell with elevated levels of hypoxia-inducible factor (HIF)-1α is more sensitive to the loss of HIF-1α than a tumour cell with lower levels of HIF-1α (for example, drug A). Second, a compound could induce cell death only when HIF-1 is elevated (drug B). This drug B paradigm is similar to the concept of synthetic lethality in yeast: when either one of two genes is altered in expression, yeast are viable; when both genes are altered in expression simultaneously, yeast are not viable. By analogy, a drug could inhibit an essential function (for example, ribosomal function) that would not cause lethality unless HIF-1 is elevated. Third, the compound could inhibit a specific HIF-1 target gene involved in cellular metabolism that is necessary for the survival of cells that have become adapted to elevated levels of HIF-1 (drug C). Fourth, the increased cytotoxicity of a compound could be the result of its activation through a HIF-1α-regulated gene (drug D). Although all of the targets depicted above are possible, the group with the most hits should be the model described by drug A. ER, endoplasmic reticulum; VHL, von Hippel-Lindau.

levels of thymidine phosphorylase, which is hypoxia responsive. As hypoxia induces an increase in thymidine phosphorylase, drugs that can be activated by its enzymatic activity will exhibit greater cytotoxicity towards hypoxic tumour cells than to non-hypoxic normal tissue.

Targeting inflammation via HIF-1

Recent work has shown that loss of HIF-1 in myeloid cells strongly inhibits, and in some cases prevents, inflammatory response, without altering the differentiation or viability of macrophage or neutrophils[110]. This activity also seems to be independent of the HIF-1 target VEGF, which was shown to be primarily a regulator of the oedema that accompanies inflammation, but was not a mediator of inflammatory cell infiltration. Given this, there could be significant opportunities to develop drugs that might prevent an inflammatory response.

One of the main targets of the HIF-1 transcription factor is the glycolytic pathway, and there is a significant and extensive literature that demonstrates that inhibition of glycolysis can prevent inflammatory response in culture systems, as well as *in vivo*. Although the notion of using metabolic inhibitors as anti-inflammatory drugs was discarded in the past, as more targeted therapeutics are developed, it could be the case that drugs that can specifically target HIF-1 can act to inhibit destructive inflammatory diseases in novel fashions. One demonstration of this potential was the experimental inhibition of a model of arthritis, in animals with a targeted deletion of HIF-1α in the myeloid lineage[110]. Models of inflammation in the context of experimental deletion of HIF-1, as well as in experiments involving HIF-1 inhibition at the pharmacological level, will allow the potential of this aspect of HIF-1 function to be exploited.

Development of HIF-inducing compounds

Although there is a great deal of interest in finding mechanisms to inhibit HIF-1 activity, there is also significant potential in inducing HIF-1 activity pharmacologically[111]. This might allow induction of angiogenesis in syndromes such as cardiovascular disease, in which that would be a desirable outcome. Wound healing, and other settings in which circulation has been interrupted, and in which formation of collateral blood vessels could be of therapeutic advantage, could conceivably benefit from the induction of HIF-1, either specifically, with targeted inducers, through gene therapy, or through the action of hypoxia mimetics. The other obvious aspect of HIF-1 induction would be mechanisms to increase the metabolic functioning of cells under hypoxia, through increases in glycolytic gene expression and other aspects of hypoxia-induced metabolic adaptation.

Induction of angiogenesis through increased HIF-1 expression might be particularly attractive insofar as it could allow not just increased expression of a single angiogenic factor or receptor, but would induce the increased expression of a range of angiogenic factors and their receptors in a coordinated fashion. A recent example of this was a study showing that introduction of an HIF-1α/VP-16 naked DNA construct allowed increased reperfusion and alleviation of ischaemia in a model of rabbit hindlimb ischaemic damage[112]. There have also been a number of interesting studies of induced expression of HIF-1α in cardiomyocytes; this is another cell type with great potential for drugs and treatments that can prevent damage caused by hypoxia and ischaemia[113–115]. Such approaches might have potential in future treatments for myocardial infarction and stroke, as well as other syndromes involving vascular occlusion.

Future directions

The presence of the HIF-1 transcription factor at the centre of many, if not most, pathways of cellular hypoxic response makes it a very attractive candidate for pharmacological manipulation. Unlike many transcription factors, the unique centrality of HIF-1, and of hypoxia, in a wide range of pathologies, make it an important target for both induction and inhibition pharmacologically. A number of methods to accomplish these aims have been devised recently, with considerable effort continuing to be focused on this topic by both academic and pharmaceutical industry laboratories. The future success of these efforts will in the end be dictated by the definition of the role of HIF-1 in the basic biology of the response to hypoxia.

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