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Natural Product-Based Inhibitors of Hypoxia-Inducible Factor-1 (HIF-1)

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Abstract

The transcription factor hypoxia-inducible factor-1 (HIF-1) regulates the expression of more than 70 genes involved in cellular adaptation and survival under hypoxic stress. Activation of HIF-1 is associated with numerous physiological and pathological processes that include tumorigenesis, vascular remodeling, inflammation, and hypoxia/ischemia-related tissue damage. Clinical studies suggested that HIF-1 activation correlates directly with advanced disease stages and treatment resistance among cancer patients. Preclinical studies support the inhibition of HIF-1 as a major molecular target for antitumor drug discovery. Considerable effort is underway, in government laboratories, industry and academia, to identify therapeutically useful small molecule HIF-1 inhibitors. Natural products (low molecular weight organic compounds produced by plants, microbes, and animals) continue to play a major role in modern antitumor drug discovery. Most of the compounds discovered to inhibit HIF-1 are natural products or synthetic compounds with structures that are based on natural product leads. Natural products have also served a vital role as molecular probes to elucidate the pathways that regulate HIF-1 activity. Natural products and natural product-derived compounds that inhibit HIF-1 are summarized in light of their biological source, chemical class, ancd effect on HIF-1 and HIF-mediated gene regulation. When known, the mechanism(s) of action of HIF-1 inhibitors are described. Many of the substances found to inhibit HIF-1 are non-druggable compounds that are too cytotoxic to serve as drug leads. The application of high-throughput screening methods, complementary molecular-targeted assays, and structurally diverse chemical libraries hold promise for the discovery of therapeutically useful HIF-1 inhibitors.

Keywords

HIF-1; Natural Product; Tumor Hypoxia; Molecular-Targeted Drug Discovery; Small Molecule HIF-1 Inhibitor; Hypoxia Selective

Introduction

In the human body, oxygen is delivered along a concentration gradient from the site of uptake in the lung capillaries to the site of consumption, where most of the oxygen is consumed by mitochondria to generate ATP [1]. Interruption of blood flow, reduction in oxygen tension, decreased oxygen carrying capacity, and failure to transport oxygen from the microvasculature to cells can all lead to an insufficient oxygen supply (hypoxia) to meet the metabolic requirements of specific tissues. In solid tumors, rapid tumor growth outstrips

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the capability of existing blood vessels to supply oxygen and hypoxic regions commonly occur in solid tumors [2–5]. Unlike normal cells from the same tissue, tumor cells are often chronically hypoxic. Hypoxia triggers tumor angiogenesis and the newly formed tumor blood vessels often fail to mature. As a result, certain tumor regions are constantly under hypoxic stress due to sluggish and irregular blood flow. The extent of tumor hypoxia correlates positively with advanced stages and poor prognosis [2–5]. Hypoxic tumor cells are more resistant than normoxic tumor cells to radiation treatment and chemotherapy and these hypoxic cells are considered an important contributor to disease relapse. Currently, the general strategies to overcome tumor hypoxia are: 1) increasing tumor oxygenation by means such as breathing carbogen (95% O_2, 5% CO_2); 2) developing chemical sensitizers to increase the sensitivity of hypoxic cells to radiation; 3) developing hypoxic cytotoxins that selectively kill hypoxic cells; and 4) developing hypoxia-selective gene therapy [2,3,5,6]. These approaches target the direct effect of hypoxia - lack of cellular oxygen. However, hypoxia also exerts indirect effects on tumor cells by inducing the expression of genes that promote hypoxic adaptation and survival. For example, hypoxia provides a physiological pressure and selects for cells with diminished apoptotic potential in oncogenically transformed cells [7].

The transcription factor that plays a critical role in hypoxia-induced gene expression is hypoxia-inducible factor-1 (HIF-1), a heterodimer of the basic helix-loop-helix (bHLH) PER-ARNT-SIM (PAS) proteins HIF-1α and HIF-1β /aryl hydrocarbon receptor nuclear translocator (ARNT) [8–10]. The HIF-1α subunit is degraded rapidly under normoxic conditions and stabilized under hypoxic conditions, while HIF-1β is constitutively expressed. In general, the availability and activity of HIF-1α protein determines the bioactivity of HIF-1. Numerous post-translational modifications are involved in regulating the stability and activity of HIF-1α protein [11–15]. In particular, oxygen-dependent prolyl hydroxylation destabilizes HIF-1α protein by promoting the tumor suppressor von Hippel-Lindau protein (pVHL)-mediated proteasomal degradation, while oxygen-dependent asparaginyl hydroxylation inactivates HIF-1α protein by preventing the interaction between HIF-1α protein and a co-activator CBP/p300 [CREB (cAMP-response element-binding protein)-binding protein/E1A-binding protein, 300 kD] [16–18]. In addition to hypoxia, other physiological and pathological factors such as cytokines, growth factors, hormones, activated oncogenes, inactivated tumor suppressors, etc., can also activate HIF-1 [11,12,19–25]. Over seventy genes are known to be regulated by HIF-1, most of these genes are regulated in a cell type-specific manner [11,19]. The list of HIF-1 target genes is still growing rapidly. Clinical studies have indicated that tumor hypoxia-associated HIF-1α overexpression correlates positively with advanced disease stages and poor prognosis among cancer patients [26–33]. Preclinical studies in numerous animal models have demonstrated that HIF-1 inhibition suppresses angiogenesis, retards tumor growth, and enhances treatment outcomes when used in combination with chemotherapeutic agents or radiation [34–43]. However, one study indicated that HIF-1α deficiency inhibits angiogenesis while promoting embryonic stem cell-derived tumor growth [44]. One recent study that used genetically engineered transformed murine astrocytes as an *in vivo* mouse model for astrocytoma suggested that the outcome of HIF-1 inhibition is dependent on the tumor microenvironment: HIF-1α deficiency retards tumor growth in the poorly vascularized subcutaneous region, while HIF-1α deficiency enhances tumor growth in the highly vascularized brain parenchyma [45]. A number of recent reviews provide an extensive overview of HIF-1 as a molecular target for cancer therapy [19–25].

A growing number of HIF-1 inhibitors of natural and synthetic origin have recently been identified. Small molecule synthetic HIF-1 inhibitors (not based on natural product or natural product-like structures) have been described in several recent reviews [19–24]. The focus of this review is natural product-based HIF-1 inhibitors and their therapeutic potential

for cancer. In general, the term "natural product" refers to low molecular weight secondary metabolites produced by animals, plants, and microbes for chemical defense and growth advantage. Natural products have been a major source of new drugs for centuries and the chemical diversity offered by natural products has not been matched by any other approach [46]. Statistics show that over 60% of the approved anticancer agents are of natural origin (natural products or synthetic compounds based on natural product models). In this review, the natural product-derived HIF-1 inhibitors are grouped by the mechanisms employed to achieve HIF-1 inhibition.

Inhibitors of HIF-1α Protein Synthesis

The availability and activity of HIF-1α protein plays an important role in HIF-1 activation. Many of the known HIF-1 inhibitors function by decreasing HIF-1α protein. The level of HIF-1α protein is controlled by an intricate balance between production and degradation. Decreased HIF-1α synthesis or increased degradation can each block the accumulation of HIF-1α protein, while the increased synthesis or decreased degradation can each induce HIF-1α protein [Fig.(1)]. The compounds that decrease HIF-1α protein synthesis may function through the following mechanisms: inhibit transcription, degrade HIF-1α mRNA, and decrease translation.

The *Streptomyces parvullus* (formerly *S. antibioticus*) metabolite actinomycin D (**1**, dactinomycin) is an inhibitor of transcription that was shown to abolish hypoxia-induced HIF-1 binding activity in Hep3B human heptoma cells [47]. A later study revealed that actinomycin D (**1**) blocked the induction of HIF-1α protein by angiotensin II (Ang II) in rat vascular smooth muscle cells but not the induction by hypoxia [48]. The HIF-1 inhibitory effects exerted by **1** may be dependent on the cell type, stimulus, and drug concentration.

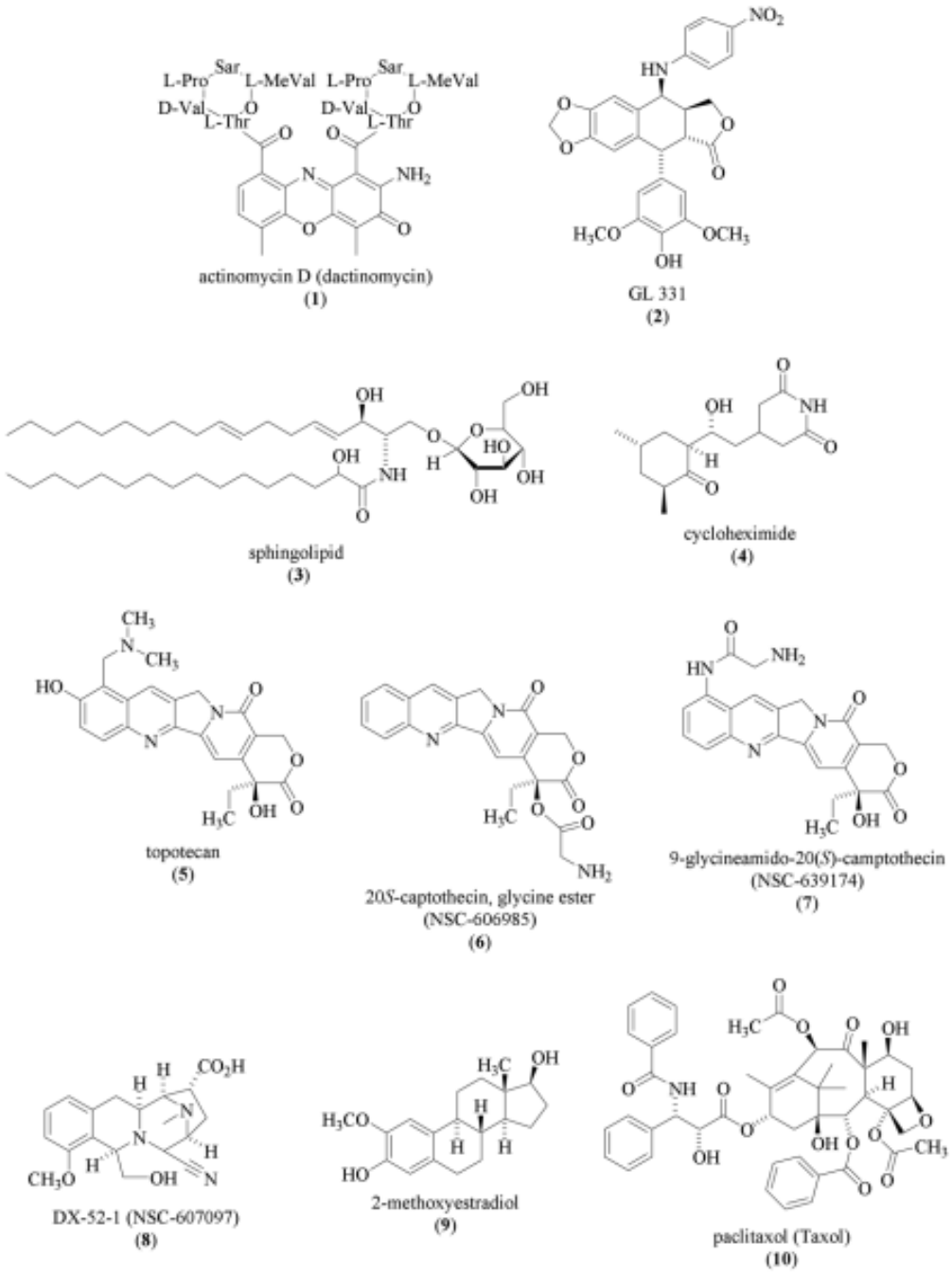
The compound GL331 (**2**) is a cytotoxic, semisynthetic podophyllotoxin-derived topoisomerase II inhibitor with IC_{50} values in the range of 0.5 to 2 μM against a panel of tumor cell lines [49]. In CL1-5 human lung adenocarcinoma cells, GL331 (10 μM) inhibited HIF-1 activation by decreasing HIF-1α mRNA level, presumably through transcriptional inhibition [50]. This inhibitory effect is unlikely to be HIF-1specific. At comparable concentrations, **2** is cytotoxic and inhibited expression of genes such as cyclin D1 in CL1-5 cells [51]. GL331 (**2**) was ineffective against gastric cancer in a clinical study [52].

Acting through yet to be determined mechanisms, a number of natural products have been shown to decrease HIF-1α mRNA levels. The Indian traditional medicine known as picroliv (a purified iridoid glycoside fraction from the roots of *Picrorhiza kurrooa*) reduced HIF-1α and vascular endothelial growth factor (VEGF) mRNA levels *in vitro* [53]. The major compounds found in picroliv are the iridoid glycosides picroside-I and kutkoside [54]. The precise chemical constituents responsible for the reported HIF-1 inhibitory activity have not been defined. When CF-1 mice were fed on a diet enriched with soy-derived sphingolipids (0.025 and 0.1%), 1,2-dimethylhydrazine-induced colonic cell tumorigenesis was suppressed [55]. At the concentrations tested, these plant 4,8-sphingadiene glucosylceramide-type sphingolipids (i.e. **3**) decreased HIF-1α mRNA levels in the intestinal mucosal cells by more than 50% [55]. The effect of plant sphingolipids on HIF-1 activation and target gene expression is not known.

The antifungal antibiotic cycloheximide (**4**, isolated from *Streptomyces griseus*) inhibits general eukaryotic protein synthesis and blocked hypoxia-induced HIF-1α protein accumulation and HIF-1 activation [8]. Compounds such as cycloheximide (**4**) and actinomycin D (**1**) are known to non-selectively inhibit gene expression, it is likely that the use of these types of natural products for their antitumor properties will always be accompanied by a considerable level of treatment-associated toxicity.

In an effort to identify small molecule HIF-1 inhibitors, a U251 human glioma cell-based high-throughput screening (HTS) assay was used to examine compounds from the National Cancer Institute (NCI) Diversity Set (~2,000 pure compounds representing the maximal three-dimensional chemical diversity) for HIF-1 inhibitory activity [56]. The compounds found to be active include three camptothecin analogues [topotecan (**5**), camptothecin 20-ester (*S*) (**6**), and 9-glycineamido-20(*S*)-camptothecin HCl (**7**)] and a quinocarmycin analogue DX-52-1 (**8**)[56]. The best characterized compound topotecan (**5**), inhibited both hypoxia (1% O_2)- and iron chelator (desferoxime; DFO)-induced HIF-1 activation at submicromolar concentrations. Topotecan (**5**) inhibits HIF-1 by decreasing HIF-1α protein translation in a topoisomerase I-dependent, oxygen-independent manner [56,57]. The indenoisoquinoline MJ-III-65 [58], a synthetic non-camptothecin Topo I inhibitor exhibited similar HIF-1 inhibitory activity as that observed for topotecan [57]. The HIF-1 inhibitory activity of topotecan is reversible and schedule-dependent *in vitro* (U251 cells), and requires a daily (not intermittent) administration schedule *in vivo* (U251 tumor xenograft model) [43]. Topotecan (**5**) is a DNA topoisomerase I inhibitor and has been used clinically as an antineoplastic agent. The therapeutic potential of topotecan (**5**) as a HIF-1 inhibitor has been recently reviewed [59].

One group of compounds reported to inhibit HIF-1α protein synthesis is microtubule disrupting agents (MDA). In human prostate PC-3 and breast MDA-MB-231 carcinoma cells, the natural estradiol metabolite 2-methoxyestradiol (2ME2, **9**) inhibited both basal and hypoxia-induced HIF-1α protein expression [60]. Mechanistic studies indicated that 2ME2 (**9**) inhibits HIF-1 by suppressing HIF-1α protein synthesis. Other MDAs that include the *Taxus brevifolia* diterpenoid taxol (paclitaxol, **10**) and the *Catharanthus roseus* (formerly *Vinca rosea*) alkaloid vincristine (**11**) also inhibited hypoxia-induced HIF-1α protein accumulation and HIF-1 activation [60]. A separate study reported that MDAs vinblastine, colchicine, and the synthetic nocodazole actually induce HIF-1α protein and activate HIF-1 in several cell lines [61]. Microtubule disruption is required for both the inhibition and the induction of HIF-1α protein [60,61]. The theory of how 2ME2 (**9**) inhibits HIF-1 has been challenged by a recent study that suggested **9** promotes HIF-1α protein degradation under hypoxia via inhibition of the mitochondrial electron transport chain (complex I) [62]. In addition, 2ME2 (**9)** did not inhibit the induction of HIF-1α protein by either *N*-mercaptopropionylglycine (2-oxoglutarate analogue) or DFO (iron chelator) in HEK 293 cells [62]. Although 2ME2 (**9**) is currently undergoing clinical evaluation for the treatment of cancer, the concentrations that inhibit HIF-1 and mitochondrial function are far above those required for the antitumor effects [60,62–64]. Whether the inhibition of HIF-1 contributes to the *in vivo* antitumor activity of 2ME2 (**9**) remains unclear.



Studies from scores of research groups have contributed to the understanding of how the phosphoinositol 3-kinase/protein kinase B (PI3K/AKT) signaling pathway regulates HIF-1α protein accumulation. These findings were summarized in a number of recent reviews [19–24,65,66]. The PI3K inhibitors wortmannin (**12**, isolated from *Penicillium fumiculosum*) and LY294002 (**13**, flavonoid-like synthetic compound) inhibited HIF-1 activation by both hypoxia and non-hypoxic stimuli via inactivation of the downstream targets that regulate the translation of HIF-1α mRNA. Two recent studies suggest that the PI3K/AKT pathway is not involved in mediating the hypoxic induction of HIF-1α protein [67,68]. The regulatory effects exerted by the PI3K/AKT pathway on HIF-1 may depend on the specific cell type and the type of stimulus. One of the protein kinases that is regulated by AKT is the

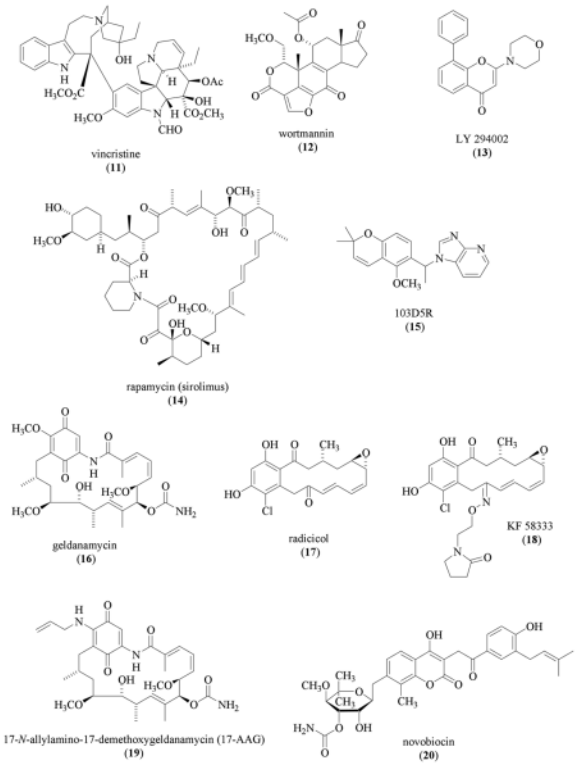
mammalian target of rapamycin (mTOR). The mTOR inhibitor rapamycin (**14**, isolated from *Streptomyces hygroscopicus*) was shown to inhibit HIF-1α protein accumulation by at least two mechanisms: inhibition of HIF-1α protein synthesis [69] and promotion of HIF-1α protein degradation [70].

A human glioma LN229 cell-based reporter assay was used to examine a combinatorial library of 10,000 "natural product-like" synthetic compounds that were based on a 2,2-dimethylbenzopyran scaffold [71]. The most active compound 103D5R (**15**) weakly inhibited HIF-1 activation by hypoxia (IC_{50} 35 μM) and more potently suppressed LN229 cell proliferation (IC_{50} 26 μM). The compound 103D5R (**15**) inhibited both hypoxia- and cobalt chloride-induced HIF-1 activation in a panel of cell lines by decreasing HIF-1α protein synthesis.

Natural Products that Promote HIF-1α Protein Degradation

Under normoxic conditions, HIF-1α protein is post-translationally modified and is rapidly degraded by the proteasome. Either a reduction in oxygen tension or the removal of enzyme cofactors can result in the stabilization of HIF-1α protein by inhibiting the enzymes that modify HIF-1α prior to proteasome degradation. Some HIF-1 inhibitors promote the degradation of HIF-1α protein in the presence of stimuli. For the purpose of this review, these types of HIF-1 inhibitors are grouped in the following text by the specific molecular target affected and the mechanism of action.

Since the first report of the interaction between HIF-1α protein and the molecular chaperone heat shock protein 90 (hsp90) [72], a number of studies have investigated the significance of this interaction using pharmacological agents that inhibit hsp90 [73–77]. Recent studies suggested that hsp90 binds to the HIF-1α PAS domains and stabilizes HIF-1α protein, and the HIF-1β /ARNT subunit competes with hsp90 for the same binding sites to stabilize HIF-1α protein [78,79]. The *Streptomyces hygroscopicus* metabolite geldanamycin (GA, **16**) inhibits hsp90 by binding to the amino-terminal ATP/ADP binding pocket [80–82]. Geldanamycin (**16**) inhibited HIF-1 activation by promoting pVHL-independent proteasomal degradation of HIF-1α protein under both normoxic and hypoxic conditions [73,75]. The GA-induced, oxygen-independent, proteasome-mediated HIF-1α protein degradation is enhanced in the absence of serum in human prostate carcinoma PC-3 and LNCaP cells [74]. Geldanamycin (**16**) also inhibited heat induced nuclear accumulation of a transcriptionally inactive form of HIF-1α protein [76]. In contrast to **16**, radicicol (**17**), another hsp90 inhibitor that also binds to the amino-terminal ATP/ADP binding pocket [83], inhibits HIF-1 in Hep3B cells by a different mechanism - blocking the binding of HIF-1 to the hypoxia-response elements (HREs) in promoters of HIF-1 target genes [77]. Using human breast carcinoma KPL-1 and KPL-4 cells as tumor models, an oxime derivative of radicicol KF58333 (**18**) was shown to decrease HIF-1α protein level *in vitro* and retard tumor growth *in vivo* [84]. Other hsp90 inhibitors that include the 17-*N*-allylamino-17-demethoxygeldanamycin (17-AAG, **19**) and the structurally unique antibiotic novobiocin (**20**, produced by *Streptomyces niveus* and *S. spheroides*) also inhibited HIF-1α protein accumulation [75,76]. Geldanamycin (**16**) is unsuitable for clinical use due to its hepatotoxicity [85]. However, the geldanamycin derivative 17-AAG (**19**) is undergoing clinical trial for the treatment of cancer [85]. In preclinical models, 17-AAG (**19**) retarded tumor growth and enhanced the therapeutic outcome of radiation treatment [85,86]. Encouraging results from drug tolerance studies were obtained in a recently completed Phase I clinical study that used 17-AAG (**19**) to treat adult patients with solid tumors [87].



Apigenin (4',5,7-trihydroxyflavone, **21**), a plant flavone found in many fruits and vegetables, has exhibited anti-tumor activity in numerous *in vitro* and *in vivo* models and is considered a chemopreventive agent for cancer [88–95]. Apigenin (100 μM, **21**) inhibited both hypoxia (5% O_2)-induced and transition metal ($CoCl_2$)-induced HIF-1α protein in Hep3B cells [96]. This study suggested that **21** may destabilize HIF-1α protein by disrupting the interaction between HIF-1α protein and hsp90. A separate study demonstrated that **21** decreased HIF-1α mRNA levels and promoted the degradation of HIF-1α protein in human ovarian carcinoma A2780/CP70 and OVCAR-3 cells, independent of the oxygen status [97]. The HIF-1 inhibitory effect exerted by **21** is most likely mediated by multiple pathways. In addition to the PI3K/AKT/p70S6K1, hsp90, and HDM2 [human ortholog of Mdm2 (mouse double minute 2 homolog)]/p53 pathways discussed in these two related HIF-1 studies, apigenin (**21**) has been reported to affect other pathways/cellular targets that range from nuclear factor-κ B (NFκ B) to human epidermal growth factor receptor 2 (HER2/neu) at comparable or lower concentrations [88–95].

Resveratrol (*trans*-3,4,5'-trihydroxystilbene, **22**) is a phytoalexin, plant metabolite produced in response to environmental stress such as fungal infection and injury. Resveratrol (**22**) has been found in various types of plants that range from grapes to peanuts [98–100]. The discovery that resveratrol inhibits a diverse set of cellular targets that are associated with the initiation, promotion, and progression of carcinogenesis [98] has sparked a tremendous amount of research on the potential of resveratrol as a cancer chemopreventive agent [99,100]. Resveratrol (**22**) was shown to inhibit basal, insulin-, and insulin-like growth factor 1 (IGF1)-induced expression of HIF-1α protein in A2780/CP70 and OVCAR-3 cells (IC_{50} values 20 to 30 μM) [101]. Resveratrol (**22**) inhibits HIF-1α protein expression by at least two mechanisms: inhibit HIF-1α protein synthesis and facilitate proteasome-mediated HIF-1α protein degradation [101]. In most of the experimental systems, **22** exerts its potential anti-tumor activities at the concentrations that range from 10 to 100 μM [99,100]. However, these concentrations (including the concentration required to inhibit HIF-1) are far

beyond what is biologically available from food consumption [99]. It would be of considerable interest to investigate if any of the metabolites formed from resveratrol (**22**) can inhibit the activation of HIF-1 or its downstream targets at biologically relevant concentrations.

Prolyl hydroxylation is one of the earlier events during the destabilization and degradation of HIF-1α protein. Overexpression of the HIF prolyl hydroxylase 3 (HPH3, PHD1, EGLN2) decreases HIF-1α protein expression and inhibits tumor growth [102]. The fungal metabolite cyclosporine (cyclosporin A or CsA, **23**, isolated from *Tolypocladium inflatum Gams*) is an immunosuppressive agent used to prevent the rejection of transplanted organs [103]. In rat glioma C6 cells, **23** (10 μM) inhibited hypoxia (2% O_2)-induced HIF-1α protein accumulation and subsequent HIF-1 activation [104]. Cyclosporine (**23**) inhibits HIF-1 activation by preventing oxygen-dependent degradation domain (ODDD)-mediated HIF-1α protein stabilization. Biochemical studies suggest that **23** stimulates the prolyl hydroxylase(s) that modifies Pro-564 of the human HIF-1α protein. However, it remains unresolved regarding which prolyl hydroxylase(s) cyclosporine (**23**) activates or by what mechanism **23** activates the enzyme.

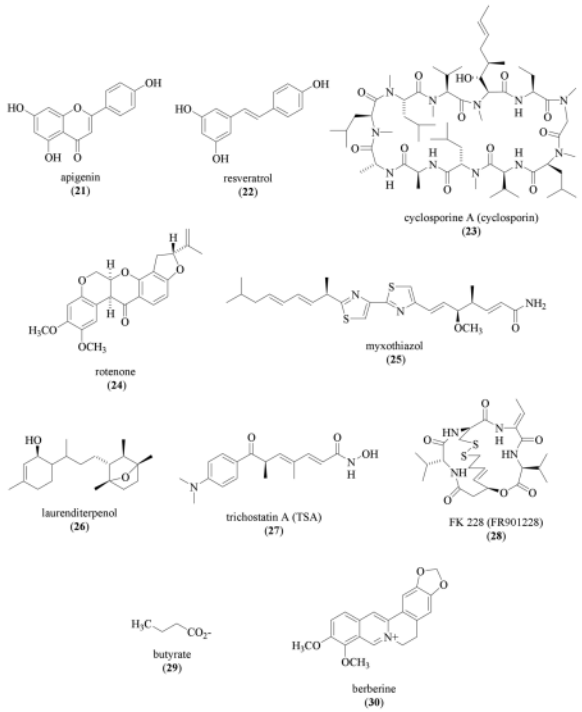
Hypoxia decreases the level of intracellular oxygen, inhibits the hydroxylases that modify HIF-1α protein, and leads to the stabilization HIF-1α protein and activation of HIF-1. The mitochondrion is the major cellular organelle that consumes oxygen. Mitochondrial inhibitors that include the plant derived natural product rotenone (**24**, isolated from *Lonchocarpus spp.*), diphenylene iodonium (DPI), myxothiazol (**25**, isolated from the myxobacteria *Myxococcus fulvus*/*Stigmatella aurantiaca*), and 4,4'-diisothiocyanatostilbene-2,2'-disulfonate (DIDS) inhibited hypoxia-induced HIF-1α protein accumulation, HIF-1 activity, and expression of HIF-1 target genes [105,106]. These compounds did not inhibit the induction of HIF-1α protein by stimuli such as DFO and $CoCl_2$ [105,106]. The mitochondrial complex I inhibitor 1-methyl-4-phenylpiridinium also inhibited hypoxic induction of HIF-1α protein [107]. Other mitochondrial inhibitors that include sodium azide, antimycin A, and cyanide have been shown to inhibit hypoxia induced HIF-1α protein accumulation [108,109]. Under hypoxic conditions, mitochondrial inhibition increases the available intracellular oxygen level, promotes HIF-1α protein prolyl hydroxylation, and is followed by enhanced degradation of HIF-1α protein under hypoxic conditions [108].

While a growing number of natural products and natural product-derived compounds have been demonstrated to inhibit HIF-1, relatively few efforts have been directed at the discovery of novel HIF-1 inhibitors from natural product-rich extracts of plants, microbes, or marine organisms as the source of chemicals. Using a T47D human breast tumor cell-based reporter assay to monitor HIF-1 activity, our research group at the University of Mississippi evaluated the lipid-soluble extracts of several thousand plants and marine organisms for HIF-1 inhibitory activity [110,111]. Bioassay-guided fractionation of the lipid extract of a Jamaican sample of the red alga *Laurencia intricata* Lamouroux (Rhodomelaceae) yielded the structurally novel diterpene laurenditerpenol (**26**) [111]. Members of the genus *Laurencia* have been shown to be a rich source of several hundred unusual secondary metabolites. Laurenditerpenol (**26**) was determined to be a structurally novel bicyclic diterpene from the analysis of high-resolution spectroscopic and spectrometric data. The absolute configuration of position C-1 was determined by analysis of the proton nuclear magnetic resonance (^{1}H-NMR) spectra of the modified Mosher ester derivatives that were prepared directly in NMR tubes. Laurenditerpenol is the first marine natural product found to inhibit hypoxia (1% O_2)-induced HIF-1 activation. In T47D cells, **26** potently inhibited HIF-1 activation by hypoxia (IC_{50} 0.4 μM) but not by the iron chelator 1,10-phenanthroline. Compound **26** inhibits HIF-1 by blocking the hypoxia-induced HIF-1α

protein accumulation. Mitochondrial respiration studies revealed that laurenditerpenol (**26**) suppresses oxygen consumption at the concentrations that inhibit hypoxia-induced HIF-1 activation. Therefore, it is believed that laurenditerpenol is the first member of a structurally novel class of marine natural product-based mitochondrial inhibitors that block mitochondrial oxygen consumption and promote the degradation of HIF-1α protein under hypoxic conditions.

Following prolyl hydroxylation, the prolyl-hydroxylated HIF-1α protein is recognized by pVHL that is part of an E3 ubiquitin ligase complex. HIF-1α protein is then polyubiquitinized and degraded by the proteosome. Certain HIF-1 inhibitors are believed to function by increasing the expression of VHL under hypoxic conditions. Hypoxia increases the expression and activity of histone deacetylases (HDAC) that down-regulate tumor suppressors p53 and pVHL and up-regulate angiogenic factors such as VEGF through the induction of HIF-1α protein and activation of HIF-1 [112]. Histone deacetylases catalyze histone deacetylation and cause chromatin remodeling. The compound (*R*)-trichostatin A (TSA, **27**), originally isolated from *Streptomyces hygroscopicus* as a fungistatic antibiotic, is a potent and specific HDAC inhibitor [113,114]. The compound TSA (**27**) was demonstrated to inhibit hypoxia-induced HIF-1α protein accumulation by inducing pVHL expression [112]. Another potent HDAC inhibitor FK228 dose-dependently inhibited hypoxia (1% O_2)-induced HIF-1α protein accumulation and the downstream events in Lewis lung carcinoma tumor model *in vitro* and *in vivo* [115]. The compound FK228 (FR901228, **28**) is a bicyclic depsipeptide produced by *Chromabacterium violaceum* that was originally isolated as an antitumor agent and later found to inhibit HDAC [116–119]. Butyrate (**29**), a weak inhibitor of HDAC, was also found to inhibit HIF-1 at millimolar concentrations [120–122]. One of the intended therapeutic applications of HIF-1 inhibitors is to augment the effects of radiation therapy. In preclinical models, HDAC inhibitors such as FK 228 (**28**) were shown to enhance radiation treatments [123,124]. A number of recent reviews have summarized the antitumor activity of HDAC inhibitors in preclinical models and their therapeutic potential for cancer [125–128]. Encouraging results have been reported from several phase I clinical trials that used FK228 (**28**) for cancer treatment [129–132].

Acetylation of HIF-1α protein at Lys-532 was reported to enhance the interaction between HIF-1α and pVHL [133]. The natural product berberine (**30**), a tautomeric alkaloid most commonly found in members of the *Berberidaceae*, was shown to inhibit hypoxia (2% O_2)-induced HIF-1α protein accumulation by promoting the lysine acetylation of HIF-1α protein [134]. The concentration of **30** (7.5 μM) that inhibited the HIF-1 pathway is equal to the cytotoxic IC_{50} value in human gastric adenocarcinoma SC-M1 cells [134]. The inhibitory effect of berberine (**30**) is unlikely to be HIF-1 specific, since **30** also inhibited other transcription factors such as AP-1 at comparable concentrations [135].



Pseudolaric acid B (**31**) is a cytotoxic diterpene isolated from *Pseudolarix kaempferi* Grord. (Pinaceae) (IC_{50} values that range from 0.02 to 1.23 μM in 39 tumor cell lines reported) [136]. In human breast carcinoma MDA-MB-468 and MDA-MB-435 cells, **31** (10 μM) inhibited hypoxia (1% O_2)-induced HIF-1α protein by promoting proteasome-mediated degradation [137]. Pseudolaric acid B (**31**) suppressed the viability/proliferation of MDA-MB-468 cells at even lower concentrations (IC_{50} 0.42 μM) [137]. Therefore, it is unlikely that the HIF-1 inhibition exerted by **31** is due to a specific effect on the pathways that regulate HIF-1. In addition to the reported cytotoxicity [136], **31** also inhibited VEGF-induced angiogenesis under normoxic conditions [137,138].

In glioblastoma multiforme cells, the Ras inhibitor *trans*-farnesylthiosalicylic acid (FTS, **32**) (70 μM) inhibited hypoxia (1% O_2) induced HIF-1α protein accumulation [139]. The observation that proteasome inhibitor MG132 blocked the FTS inhibition on HIF-1α protein suggests that **32** promotes HIF-1α protein degradation. The compound FTS (**32**) was shown to inhibit Ras downstream target the PI3K/AKT/mTOR pathway, but not the mitogen-activated protein kinase kinase/extracellular signal-regulated kinase (MEK/ERK) pathway. As previously discussed, the PI3K/AKT/mTOR pathway regulates the synthesis of HIF-1α protein. The mechanism by which *trans*-farnesylthiosalicylic acid (**32**) promotes HIF-1α protein degradation is not clear.

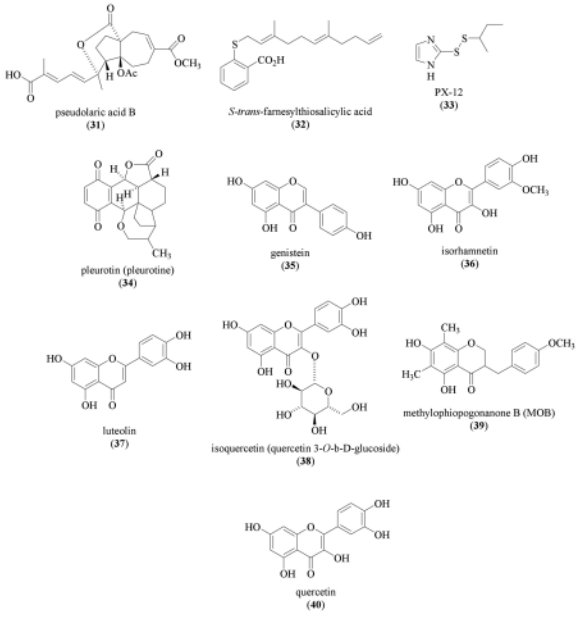
Compounds that Prevent HIF-1α Protein Accumulation

The following section of this review focuses on the compounds that inhibit HIF-1α protein accumulation with yet to be identified molecular mechanisms.

Early studies on HIF-1 implicated that cellular redox status regulates HIF-1 activity [140]. At the molecular level, redox proteins such as redox factor 1 (REF-1) and thioredoxin upregulate HIF-1 activity by enhancing the interaction between HIF-1α and transcriptional coactivators CBP/p300 and SRC-1 [141–143]. Overexpression of thioredoxin-1 in MCF-7 human breast tumor cells induced the accumulation of HIF-1α protein, HIF-1 activation, and

expression of HIF-1 target genes without affecting the level of HIF-1α mRNA [144]. The compounds PX-12 (1-methylpropyl 2-imidazoyl disulfide, **33**), an inhibitor of thioredoxin-1, and pleurotin (**34**), a toxic antibiotic obtained from the mushroom *Pleurotus griseus* that acts as an inhibitor of thioredoxin-1 reductase, each inhibited HIF-1α protein accumulation under both normoxic and hypoxic conditions [145]. In MCF-7 cells, PX-12 (**33**) and pleurotin (**34**) inhibited hypoxia-induced HIF-1α protein accumulation (IC_{50} values 7.2 and 7.6 μM, respectively); and suppressed cell proliferation/viability (IC_{50} values 1.9 and 0.9 μM, respectively). Similar HIF-1 inhibitory effects of **33** and **34** were also observed in HT-29 human colon carcinoma cells and pVHL-mutated RCC4 renal cell carcinoma cells. The compound **33** also inhibited the expression of HIF-1α protein in MCF-7 tumor xenografts. How **33** and the natural product pleurotin (**34**) decrease HIF-1α protein level is unclear. The compound PX-478 (*S*-2-amino-3-[4'-*N*,*N*-bis(2-chloroethyl)amino] phenyl propionic acid *N*-oxide dihydrochloride) was shown to decrease HIF-1α protein accumulation under both normoxic and hypoxic conditions *in vitro*, and in various tumor xenograft models [145]. The antitumor effects of PX-478 correlate directly with the level of tumor HIF-1α protein.

The plant isoflavone genistein (**35**), a broad-spectrum tyrosine kinase inhibitor, inhibits HIF-1 by blocking the induction of HIF-1α protein [146,147]. Subsequent studies have shown that several additional flavonoids and homoisoflavonoids are weak inhibitors of hypoxia-induced HIF-1 activation in a CHO (A4-4 clone) cell-based reporter assay [148]. Isorhamnetin (**36**), leuteolin (**37**), isoquercetin (**38**), and methylophiopogonanone B (**39**) inhibited HIF-1 activation at concentrations that range from 3 to 9 μg/mL (approximately 30 to 90 μM). At even higher concentrations (> 27 μg/mL), quercetin (**40**) also inhibited HIF-1 activation. The precise IC_{50} values were not determined. The compound MOB (**39**) was also shown to inhibited HIF-1 by blocking the induction of HIF-1α protein.



The antitumor nucleoside analog 9-β-D-arabinofuranosyl-2-fluoroadenine (F-ara-A, **41**) interferes with DNA replication and inhibits VEGF expression in ovarian cancer cells (OVCAR-3 and A2780/CP70) [149]. The compound F-ara-A (**41**) significantly inhibited HIF-1α expression at concentrations that ranged from 2.5 to 10 μM [149]. The observation that AKT activation overcomes the inhibition by F-ara-A (**41**) suggests that **41** inhibits HIF-1 through the PI3K/AKT signaling pathway. Thymidine phosphorylase (TP; also known as platelet-derived endothelial growth factor or PD-ECGF) stimulates endothelial

cell chemotaxis and is essential for angiogenesis [150]. Thymidine phosphorylase catalyzes the degradation of 2-deoxy-D-ribose-1-phosphate to 2-deoxy-D-ribose (**42**). This thymidine metabolite **42** (10 μM) was shown to produce a slight decrease in hypoxia-induced HIF-1α in HL-60 human leukemia cells [151]. The effect of **42** on either HIF-1 activation or HIF-1 target gene expression was not determined. In an unrelated study, the natural opium alkaloid morphine (**43**) was reported to inhibit HIF-1α protein expression in myocardial ischemia models *in vitro* and *in vivo* with a yet to be identified mechanism [152].

In order to discover novel, potent, and hypoxia-selective HIF-1 inhibitors, our research group initiated a natural product drug discovery effort that combines a traditional natural product-based chemical approach with molecular-targeted screening assays [110,111]. The lipid extract of the aquatic plant *Saururus cernuus* L. (Saururaceae) potently inhibited hypoxia-induced HIF-1 activation in the primary assay [110]. *Saururus cernuus* (also known as “lizards tail”) is a native aquatic plant found in the eastern United States that has a long history of medicinal use by both Native Americans and colonists, including the treatment of tumors [153,154]. Bioassay-guided chromatographic fractionation of the *S. cernuus* organic extract resulted in the isolation of manassantin B (**44**) and 4-*O*-demethylmanassantin B (**45**) [110]. From spectroscopic analysis of the Mosher MTPA ester derivatives of manassantin B, this study reported for the first time the absolute configurations of the chiral centers in each side chain of the representative manassantin-type dineolignans. Both **44** and **45** are among the most potent small molecule HIF-1 inhibitors discovered (IC_{50} values 3 and 30 nM, respectively). The manassantins selectively inhibited the activation of HIF-1 by hypoxia. Even at significantly higher concentrations (IC_{50} values >100 and 1000 nM, respectively), compounds **44** and **45** did not inhibit the activation of HIF-1 by the iron chelator 1,10-phenanthroline. Both compounds also inhibited the induction of the angiogenic factor VEGF by hypoxia. Further study revealed that manassantin B (**44**) selectively blocked the induction of HIF-1α protein, the oxygen-regulated HIF-1 subunit that determines HIF-1 activity.

Inhibitors of the Interaction between HIF-1α and Coactivators

The following section of this review discusses HIF-1 inhibitors that inhibit HIF-1 activation without decreasing the level of HIF-1α protein.

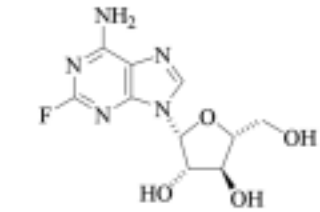
The observation that PD98059 (**46**) suppressed HIF-1 target gene expression suggested that the mitogen-activated protein kinase (MAPK) pathway regulates HIF-1 activity [155]. The synthetic compound PD98059 (**46**) is based on a flavone natural product-like structure that specifically inhibits MEK1 [156,157]. Activation of the MEK1/p42/p44 MAPK enhances HIF-1 activity and **46** inhibits HIF-1 activation [158,147]. The compound PD98059 (**46**) inhibits the activation of MAPK that enhances the interaction between the HIF-1α C-terminal transactivation domain (CTAD) and p300 without affecting HIF-1α protein level or the binding between HIF-1 and HRE [147,159,160].

After establishing that blocking the interaction between HIF-1α and p300/CBP proteins suppresses tumor growth in xenograft models, Kung and coworkers took a high-throughput screening (HTS) approach to discover small molecule disruptors of the HIF-1α/p300 interaction [38,161]. Using a time resolved fluorescence primary assay that monitors the interaction between the human HIF-1α CTAD domain (786–826) and the p300 CH1 domain (302–423) followed by secondary assays, the fungal natural product chetomin (**47**, isolated from *Chetomium* spp.) was identified as a disruptor of HIF-1α/p300 interaction from a library of >600,000 natural and synthetic compounds [161]. Chetomin (**47**), which was originally named "chaetomin," is an antibiotic isolated from *Chaetomium cochliodes* by Waksman and coworkers in 1944 [162,163]. Due to its complex nature, the chemical structure of **47** was not elucidated until decades later [164,165]. Chetomin (**47**) binds to the

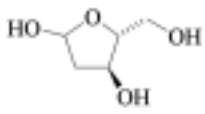
CH1 domain of p300 and disrupts the tertiary structure [161]. At submicromolar concentrations, **47** inhibited both hypoxia- and iron chelator-induced HIF-1 activation and its downstream targets *in vitro* as well as *in vivo*. Toxicity associated with the administration of chetomin (**47**) in animal models may limit its therapeutic potential for use in treating cancer.

Other HIF-1 Inhibitors

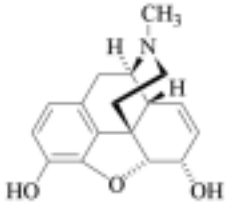
The protein kinase C (PKC) inhibitor 7(*R*)-hydroxystaurosporine (UCN-01, **48**) was originally isolated from cultures of a *Streptomyces* sp. [166]. Compound **48** inhibits endothelial cell proliferation (human aortic endothelial cells; HAEC) at considerably lower concentrations (IC_{50} 32 to 75 nM) than the concentrations required to suppress tumor cell lines (i.e. LNCaP cell IC_{50} 1000 nM) [167]. Figg and coworkers demonstrated that **48** significantly decreases hypoxia-induced and desferoxamine-induced HIF-responsive promoter activity in human prostate tumor PC3M cells. The IC_{50} was not determined. Examination of the data reported would indicate that the IC_{50} value of **48** is between 40 and 200 nM. The effect of UCN-01 (**48**) on HIF-mediated gene expression was not determined.



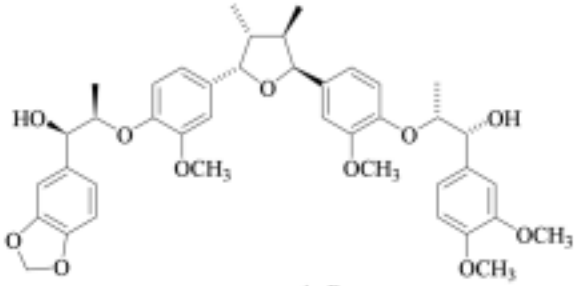
F-ara-A (2-fluoro Ara-A; fludarabine)
(**41**)



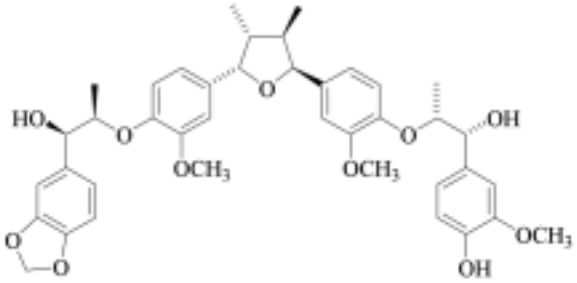
2-deoxy-D-ribose
(**42**)



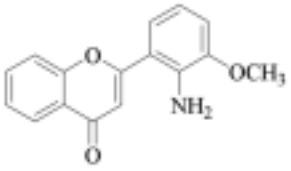
morphine
(**43**)



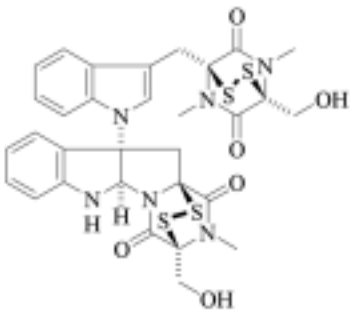
manassantin B
(**44**)



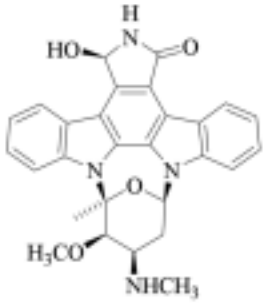
4-*O*-demethylmanassantin B
(**45**)



PD 98059
(**46**)



chetomin (cheatomin)
(**47**)



7(*R*)-hydroxystaurosporine (UCN-01)
(**48**)

Perspectives

Since oxygen is critical to our survival, it is not surprising to see that many toxins and other natural products inhibit HIF-1, a key regulator of oxygen homeostasis. Distinctly different signaling pathways regulate the availability and activity of HIF-1 in response to environmental, extracellular, and intracellular stimuli. While hypoxia appears to be a universal stimulus for HIF-1 activation, other stimuli act in a cell-type specific fashion. Correspondingly, certain HIF-1 inhibitors are more selective towards particular stimuli, while others act as more generalized inhibitors. Many drug discovery efforts are limited by bioassays that only monitor a narrow set of HIF-1 regulatory pathways. It may be necessary to incorporate both environmental and genetic factors into the *in vitro* model(s) when developing bioassays to screen chemical libraries for HIF-1 inhibitors. Since hypoxic tissues are intrinsically undervascularized, drug delivery will remain a critical factor that can hinder or contribute to the clinical success of HIF-1 inhibitors.

In general, the premise behind molecular-targeted drug discovery is the idea of identifying new therapeutically useful agents that selectively target a disease-specific molecular mechanism(s) or pathway(s). In terms of antitumor drug discovery, the goal of such efforts is to discover new chemotherapeutic agents that are directed only at tumor cells and would not cause general cytotoxicity-related side effects [168]. The discovery of new natural product-based inhibitors of HIF-1 holds, such promise. Therefore, it is surprising to see that so much effort in the discovery of HIF-1 inhibitors is currently spent characterizing small molecules that inhibit HIF-1 at cytotoxic concentrations [summarized in Table (1), reference 22]. It is possible that the ability of certain cytotoxic compounds to inhibit HIF-1 may provide some degree of selectivity to otherwise nonspecific cytotoxins. Many of the currently used chemotherapeutic drugs such as alkylating agents and antimitotics rely on relatively non-selective cytotoxic mechanisms. The fact that some of the compounds described as HIF-1 inhibitors inhibit HIF-1 at concentrations much higher than those required to inhibit proliferation and kill cells, does bring in the question as to the biological relevance of such studies. Efforts to discover therapeutically useful HIF-1 inhibitors, including natural product-based inhibitors, should include an early stage deselection criteria that exclude cytotoxic compounds. Molecular-targeted drug discovery programs that fail to consider the cytotoxic properties of the active compounds identified are destined to continue producing relatively nonselective cytotoxic antitumor agents. On the other hand, programs that combine well-designed bioassays and unique sources of chemical diversity, such as natural products, will have great potential to discover tumor-specific chemotherapeutic agents.

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List of Abbreviations

17-AAG	17-*N*-allylamino-17-demethoxygeldanamycin
2ME2	2-methoxyestradiol
AKT	Protein kinase B
Ang II	Angiotensin II
ARNT	Aryl hydrocarbon receptor nuclear translocator
bHLH	Basic helix-loop-helix
CBP	CREB (cAMP-response element-binding protein)-binding protein
COX-2	Cyclooxygenase-2
CsA	Cyclosporine (same as cyclosporin A)
CTAD	C-terminal transactivation domain (same as TADC)
DFO	Desferoxamine
DIDS	4,4’-diisothiocyanatostilbene-2,2’-disulfonate
DPI	Diphenylene iodonium
ERK	Extracellular signal-regulated kinase (same as MAPK)
FTS	S-*trans*-farnesylthiosalicylic acid [same as (*E*,*E*)-*S*- farnesylthiosalicylic acid]

GA	Geldanamycin
HAEC	Human aortic endothelial cells
HER2/neu	Human epidermal growth factor receptor 2 (
HDAC	Histone deacetylases
Hdm2	Human ortholog of Mdm2 (Mouse double minute 2 homolog)
HIF-1	Hypoxia-inducible factor-1
HPH	HIF prolyl hydroxylase (same as PHD)
HRE	Hypoxia response element
hsp90	Heat shock protein (90 kD)
HTS	High-throughput screening
MAPK	Mitogen-activated protein kinase (same as ERK)
MDA	Microtubule-disrupting agent
MEK	Mitogen-activated protein kinase kinase (same as MAPKK; same as MAPK/ERK kinase)
mTOR	Mammalian target of rapamycin
NCI	U.S. National Cancer Institute
NFκB	Nuclear factor-κB
NMR	Nuclear magnetic resonance
ODDD	Oxygen-dependent degradation domain
PAS	PER-ARNT-SIM
p300	E1A-binding protein, 300 kD
PHD	Prolyl hydroxylase domain-containing protein (same as HPH)
PI3K	Phosphoinositol 3-kinase
PKC	Protein kinase C
REF-1	Redox factor 1
TP	Thymidine phosphorylase (same as platelet-derived endothelial growth factor or PD-ECGF)
TSA	(*R*)-trichostatin A
UCN-01	7(*R*)-hydroxystaurosporine
VEGF	Vascular endothelial growth factor
VHL	von Hippel-Lindau disease tumor suppressor

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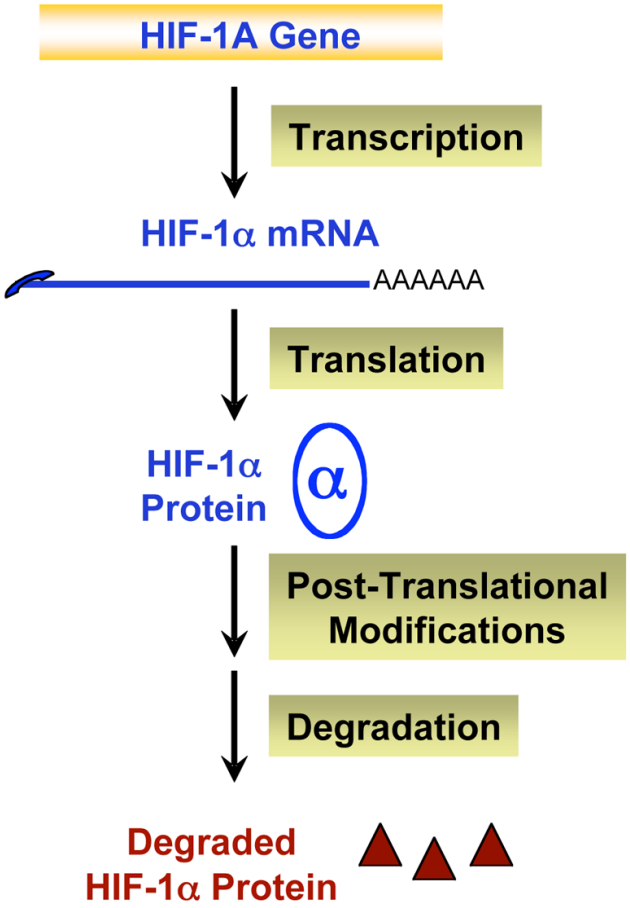

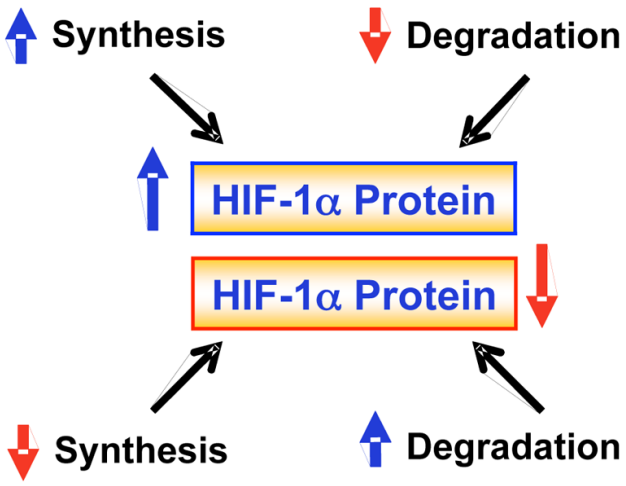

Figure 1.