Review Article

Histone Deacetylase Inhibitors: The Epigenetic Therapeutics That Repress Hypoxia-Inducible Factors

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Histone deacetylase inhibitors (HDACIs) have been actively explored as a new generation of chemotherapeutics for cancers, generally known as epigenetic therapeutics. Recent findings indicate that several types of HDACIs repress angiogenesis, a process essential for tumor metabolism and progression. Accumulating evidence supports that this repression is mediated by disrupting the function of hypoxia-inducible factors (HIF-1, HIF-2, and collectively, HIF), which are the master regulators of angiogenesis and cellular adaptation to hypoxia. Since HIF also regulate glucose metabolism, cell survival, microenvironment remodeling, and other alterations commonly required for tumor progression, they are considered as novel targets for cancer chemotherapy. Though the precise biochemical mechanism underlying the HDACI-triggered repression of HIF function remains unclear, potential cellular factors that may link the inhibition of deacetylase activity to the repression of HIF function have been proposed. Here we review published data that inhibitors of type I/II HDACs repress HIF function by either reducing functional HIF-1α levels, or repressing HIF-α transactivation activity. In addition, underlying mechanisms and potential proteins involved in the repression will be discussed. A thorough understanding of HDACI-induced repression of HIF function may facilitate the development of future therapies to either repress or promote angiogenesis for cancer or chronic ischemic disorders, respectively.

1. Introduction

Tumors are one of the leading causes of disability and mortality in the USA and other developed countries. While many advances have been made in both basic research and clinical treatment, the development of more efficient cancer-specific therapies remains an unfinished mission. In addition to surgery and radiation therapy, chemotherapy is an important component in treating a variety of cancers, particularly for late stage, advanced cancers that are unsuitable for surgical removal. Chemotherapeutics are commonly antiproliferative compounds that preferentially kill dividing cells, rarely discriminating cancer cells, or normal dividing cells such as hematopoietic cells. Given sufficient dose and time, chemotherapeutics should be able to kill all cancer cells theoretically. However, in clinical practice, two of the major hurdles of chemotherapy are (1) tumor hypoxia, which is related to inefficient drug delivery and triggers drug resistance [1] and (2) adverse effects on normal tissues, which frequently limit the dose and duration of treatment. These two hurdles limit the efficacy of chemotherapy. To overcome these hurdles, an emerging trend in cancer therapy is to specifically target hypoxic cancer cells [1, 2]. Indeed, hypoxia, HIF activation, and angiogenesis in solid tumors have been demonstrated by many independent studies [3–5]. Particularly, hypoxic and angiogenic tumors are usually resistant to traditional radiation and chemotherapy [6–10]. Blocking tumor angiogenesis has been extensively explored as a novel treatment for cancers in the past decade. The identification of HIF-function as the master regulator of angiogenesis and tumor cells adaptation to various stress conditions, including those caused by chemotherapy and radiation, provides the rationale to target HIF function as an important part in cancer therapy. Since HIF function is
Table 1: Histone deacetylases: Classification and characteristics.

| Classes | HDACs | Localization | Features |
|---------|-------|--------------|----------|
| Class I | Ia: HDAC 1, 2 | Nucleus | Related to yeast RPD3 deacetylase Zinc dependent [57] |
|         | Ib: HDAC 3 | Nucleus & cytoplasm | |
|         | Ic: HDAC 8 | Nucleus | |
| Class II| Ia: HDAC 4, 5, 7, 9 | Nucleus/cytoplasm [58] | Related to yeast Hdal Zinc-dependent [23, 24] |
|         | Iib: HDAC6 | Cytoplasm | |
|         | Iic: HDAC10 | Nucleus/cytoplasm [59, 60] | |
| Class III | Sirtuins (Sirt1-7) | Nucleus, cytoplasm & mitochondria | Related to the Sir2 NAD+-dependent [25] |
| Class IV | HDAC 11 | Nucleus & cytoplasm | Features of both classes I and II [61], Zinc independent |

essential for both tumor progression and tissues’ adaptation to chronic ischemia, it is a potential therapeutic target not only for cancer but also for chronic ischemic disorders.

In recent years, several HIF inhibitors have been identified by compound screening processes [11–13]. Interestingly and surprisingly, basic research and clinical trials have shown that HDACIs block angiogenesis and suppress tumor growth [14–16]. It has been gradually realized that these effects are at least partially mediated by repressing HIF function. Specifically, a unique phenomenon has been reported that inhibitors of class I/II HDACs, which usually stimulate transcription factors, repress the transactivation potential of both HIF-1α and HIF-2α [17]. Importantly, HDACIs repress HIF-α in all cells examined, indicating a ubiquitous mechanism [17, 18]. Although HDACIs were originally designed as epigenetic therapeutics, the effects of these compounds are generally pleiotropic. The direct molecular targets of HDACIs and the biochemical mechanisms underlying the repression of HIF function remain elusive. In this paper, we will first briefly summarize HDACs, HDACIs, and the regulatory mechanisms of HIF function. We then will focus on analyzing the potential links between protein hyperacetylation triggered by inhibitors of type I/II HDACs and its repressive effect on HIF function.

2. Histone Deacetylases and Histone Deacetylase Inhibitors

HDACs compass a large family of enzymes that remove the acetyl groups from N-ε-lysines of histones [19–21]. Since the original discovery of histone acetylation, nonhistone proteins such as transcription factors or coactivators have been shown to be subjective to the same modification. Therefore, HDACs are now redefined as lysine deacetylases to more precisely reflect the fact that its substrates, acetylated lysyl residues, are not exclusive for histones [22]. Acetylation status of these proteins is usually reversibly regulated by a dynamic balance between acetyl transferases (HATs) and HDACs.

So far 18 HDACs have been identified from mammalian cells, which are classified into four classes based on their homology to yeast enzymes [23–25] (Table 1). HDAC1-3, 8 are nuclear localized class I HDACs and are most commonly associated with transcription regulatory complexes known as Sin3, NuRD, CoRest (HDAC1, 2), and SMRT/NCoR (HDAC3) [19–21, 26, 27]. Generally, Class I HDACs are considered to be repressive factors for gene expression, despite a few exceptions [28, 29]. HDAC1 is also known to inhibit the function of the phosphatase PTEN involved in cell signaling by deacetylation [30]. HDAC3 is reported to control the acetylation of p65, the subunit of NF-κB, which is a key transcription factor involved in responses to inflammation and other cellular stresses [31]. HDAC4–7, 9, 10 belong to class II [20]. HDAC4 is involved in a multiprotein transcriptional corepressor complex and is implicated in myocyte differentiation, skeletogenesis, and neuronal survival [32–35]. HDAC5 has been suggested to interact with nuclear receptor corepressors 1 and 2, which are important in the down regulation of gene expression [36]. A key role in development and pathophysiology of cardiomyocytes has been proposed for HDAC5 [37, 38]. As an exclusive member mainly functioning in the cytoplasm, HDAC6 deacetylates cytoplasmic nonhistone substrates including Hsp90 [23, 39] and α-tubulin [40–43]. HDAC6 also binds to misfolded proteins and dynein motors, thus allowing the misfolded proteins to be physically transported to molecular chaperones and proteasomes for degradation [44]. Class III comprises of the NAD+-dependent Sirt1-7 [45–49], which has been implicated in caloric restriction, aging, neuronal degeneration, and longevity [50–52]. HDAC11, which is sometimes called class IV [53], negatively regulate interleukin 10.

HDAC inhibitors (HDACI) encompass several diverse compounds that inhibit deacetylases. Several HDACIs commonly seen in literatures are listed in Table 2, and the chemical structures of representative compounds are shown in Figure 1. Since protein acetylation \textit{in vivo} is, in most cases, reversibly regulated by a dynamic balance between histone acetyl transferases (HATs) and HDACs [19–21], exposure of cells to HDACIs breaks the balance and induces hyper-acetylation of proteins. Similar to enhanced HAT activity, HDACIs generally promote gene expression by elevating the acetylation status of histones, transcription factors, and coactivators. Importantly, HDACIs are anticaner compounds undergoing intensive investigation; some of them have been approved by the US Food and Drug Administration (FDA) for clinical treatment of certain types of cancer patients. Clinical and experimental data show that inhibitors of class I/II HDACs repress tumor growth and induce apoptosis.
While mainly considered as epigenetic therapeutics, HDACIs enhance the level of acetylation of nonhistone proteins as well. For example, the acetylation states of the transcription regulators such as c-Myb, E2F1, HNF-4, Ku70, NF-κB, p53, RB, Runx, Sp3, STATs, and YY1 are affected by HDACIs [14, 54]. It is important to note that HDACIs may also affect the acetylation of cytoplasmic/mitochondrial proteins that are not directly involved in the transcriptional control of gene expression [55, 56].

3. Hypoxia, Hypoxia Inducible Factors, the Oxygen Sensing Pathway, and Angiogenesis

The oxygen-sensing pathways, which represent the canonical regulatory mechanism of HIF function, have been investigated in depth, making it possible to modulate HIF function as a novel therapy. Hypoxia-inducible factors (HIF-1, 2, 3) are heterodimeric transcription factors, each composed of a unique α-subunit (HIF-1α, 2α or 3α) and a common β-subunit (HIF-β) shared by HIF-α and other transcription factors. HIF-1 and HIF-2 are the major contributors to the transcription of HIF target genes that encompass several orchestrated functional groups [69, 70]. While regulating the expression of overlapping target genes, HIF-1 and HIF-2 have been demonstrated to possess distinct nonredundant functions [71–73]. The overall biological effect of expression of HIF target genes is to facilitate the utilization of oxygen and other nutrients, thus inducing cellular adaptation to hypoxia, chemotherapy, and other cellular stresses [69, 70]. Most importantly, the expression of key proangiogenic factors, such as vascular endothelial growth factor (VEGF) [74, 75], bFGF, and their receptors [76, 77], stimulates angiogenesis and vasculogenesis, which are fundamental processes involved in tumorigenesis, wound healing, chronic ischemic adaptation, and early embryonic development.

As heterodimeric transcription factors, HIF-1 and HIF-2 are functionally controlled by their alpha subunits (HIF-1α and HIF-2α, resp., HIF-α collectively). HIF-α activity is controlled by two well-known mechanisms [78–80] (Figure 2). Firstly, HIF-α is rapidly degraded through a hydroxylation-ubiquitination-proteasomal system (HUPS) when oxygen is sufficient. With an adequate oxygen supply, HIF-α is hydroxylated at two prolyl residues in the oxygen-dependent degradation domain (ODD) by a family of prolyl hydroxylases (PHD) [78–80]. The oxygen-facilitated hydroxylation makes HIF-α recognizable by VHL, an E3 ligase, for ubiquitination [78–80]. Consequently, the ubiquitinated HIF-α is degraded by the proteasome system [81, 82]. Secondly, HIF-α activity is controlled by its transactivation potential (TAP), which is in turn controlled by its interaction with coactivator p300/CBP and other factors [83, 84]. Factor inhibiting HIF-1 (FIH), an oxygen-dependent hydroxylase, modifies an Asn residue at the carboxyl terminal activation domain (HIF-αCAD) and disrupts its interaction with p300/CBP [85, 86]. In addition, HIF-α has an N-terminal activation domain (NAD) whose activity is also influenced by oxygen availability. Lack of oxygen (hypoxia, anoxia), a common pathophysiological condition frequently complicated with neoplastic, cardiovascular, hematologic, and respiratory disorders, represses the activity of hydroxylases and activates HIF function [78–80]. Finally, hypoxia triggered generation of reactive oxygen species by the mitochondrial electron transfer chain has been identified as a major player in the stabilization of HIF-α [87, 88]. Oxygen and oxygen-dependent hydroxylation-triggered events form the conventional regulatory pathways of HIF function (Figure 2), illustrating a physiological feedback.

HIF activation and the expression of HIF target genes play key roles in tumorigenesis and angiogenesis. One of the major metabolic features of tumors is that they usually demand increased oxygen, carbon, and nitrogen sources because of active biosynthesis during cell growth and proliferation [89]. Continuous growth of primary or metastatic tumors can happen only when this demand is met, usually by angiogenesis. Indeed, hypoxia and HIF-1 activation has been observed in a variety of solid human tumors [90–93], accompanied by overexpression of HIF target genes and angiogenesis. Loss of HIF-1α dramatically retards solid tumor growth in vivo and is correlated with a reduced capacity to release proangiogenic factors [94–96]. Angiogenesis is defined as the formation of new blood vessels from pre-existing vessels. Neoplastic angiogenesis involves three major components [97]: (1) the tumor cells that synthesize and secret signaling molecules and growth factors (paracrine), (2) the extracellular matrix and surrounding microenvironment, and (3) the responses of endothelial and other stromal cells. It is noted that tumor-secreted signaling molecules not only function on endothelial cells,
Figure 1: Chemical Structures of Representative Inhibitors of Class I/II HDACs. While structurally diverse, they share one common feature: the existence of active groups targeting the zinc-dependent catalytic sites of class I/II HDACs.

but also satisfy the growth factor requirement of tumor cells. The HIF-stimulated autocrine loop renders tumor cells independent of growth factors from other origins. Therefore, HIF-induced angiogenesis and secretion of growth factors fulfill the major needs of sustainable tumor growth, invasion, and progression. While expression of HIF-1α and HIF-2α has been observed in both stromal and tumor cells [71–73, 98, 99], an intrinsic or autonomous role of HIF-2α in endothelial cells has been also reported [72]. HIF-1α is believed to play more important roles in regulating tumor cell survival and metabolic reprogramming in response to hypoxia [94–96].

Hypoxia is not the only cause of HIF activation in tumors. In solid tumors, in addition to hypoxia, a combination of extracellular and intracellular factors (Figure 3), including growth factors, mitogenic signaling (MAPK, PI3K/Akt), activation of oncogenes, and loss of tumor suppressors (VHL, p53 and PTEN), activates HIF-α by acting on various points of the canonical pathways [78–80, 100–106]. Considering the complexity of signaling pathways that lead to HIF-activation in tumors, it is generally difficult to repress HIF function by repairing the aberrant canonical pathways. Furthermore, the diverse contributions of HIF target genes to metabolic reprogramming, cell survival, tumor growth, and progression make it less effective to block each effect of HIF activation. Instead, directly targeting HIF may be an ideal strategy for cancer therapy. Currently, many studies are actively exploring compounds to directly repress HIF-α, and several HIF inhibitors have been developed [11–13].

Interestingly, some small molecular weight compounds under development for cancer therapy but not originally intended to target HIF function show good anticancer effects and antiangiogenic features. These include HDACIs [14–16], heat shock protein (HSP) 90 inhibitors [107, 108], proteosome inhibitors [109–112] and microtubule inhibitors [113–115]. While these compounds are aimed at distinct cellular targets, studies have linked their antiangiogenic and antitumor effects to HIF inhibition.

4. Histone Deacetylase Inhibitors Repress Tumor Angiogenesis and HIF Function

Accumulating evidence suggests that inhibition of class I/II HDAC activity represses HIF function in tumor cells [17, 18, 116–122]. The HDACIs showing anti-HIF activity generally block class I and II HDACs. While most inhibitors of class I/II HDACs are not selective for a particular deacetylase, they do not directly repress the enzymatic activity of class III HDACs (Sir2 family) [48, 49, 123]. Trichostatin A (TSA) is among several HDACIs reported to repress angiogenesis in vitro and in vivo [118, 124]. Other HDACIs including FK228 (depsipeptide, FR901228) [120, 121], butyrate [28, 122], and LAQ82481 have been known to repress angiogenesis and expression of HIF regulated pro-angiogenic factors, such as vascular endothelial growth factor (VEGF). While HIF1 had been accepted as a major regulator of angiogenesis, the early explanations for the antiangiogenesis effects of HDACIs...
Figure 2: Regulation of HIF-1α by Oxygen-Dependent Hydroxylation. HIF function is continuously regulated by the concentration of molecular oxygen, representing an essential part of physiological feedback loop. In this feedback loop, oxygen sensing is achieved by oxygen-dependent hydroxylation of specific amino acid residues of HIF-α. Hydroxylation of two prolyl residues leads to ubiquitination and proteasome-dependent degradation of HIF-α. Hydroxylation of an asparagine residue located at the CAD by FIH impairs its interaction with coactivator p300 or CBP, thus repressing the transactivation activity. Note that the hydroxylation reactions require ferrous ion and ascorbic acid as cofactors, and 2-oxoglutarate as cosubstrate.

Figure 3: Multiple Signaling Pathways Regulate HIF Function and Key Determinants of the Transcription Activity of HIF-1 and HIF-2. The overall function of HIF complexes is mainly determined by protein levels of the α-subunits (HIF-α) and their interactions with p300 or CBP. Multiple signaling pathways may modulate HIF function either through acting on HIF-α (stability or simple posttranslational modifications) or on p300/CBP (posttranslational modifications). Eventually, the HIF-α-p300 or HIF-α-CBP complexes serve as the integrators of these signals and coordinate the dynamic reprogramming of gene expression. HDACs may directly interact with the HIF transcription complexes, or indirectly or functionally interact with these signaling pathways and regulate HIF function.

varied, perhaps because HDACIs have a pleiotropic nature, and multiple pathways regulate angiogenesis. Later findings suggest that HDACI-mediated repression of angiogenesis renders its effect on HIF function in tumor cells [118, 120, 124–127]. While detailed molecular and biochemical mechanisms remains unknown, several current explanations include (1) HDACI-mediated destabilization of HIF-1α [18–21, 26–29, 40–43, 48, 49, 53, 116, 118, 123, 124, 128], (2) HDACI-mediated repression of the transactivation potential of the carboxyl-terminal transactivation domain of HIF-αCAD [17, 129], (3) repressing DNA binding ability [120], and (4) inhibiting nuclear translocation of HIF-1 [117, 122]. Below we will focus our discussion on HDACI-mediated destabilization of HIF-1α and HDACI-mediated repression of HIF-αCAD TAP, the two better supported models. We will discuss data consistent with or contrary to these views. Interested readers are referred to other proposed mechanisms including inhibiting nuclear translocation of HIF-1α [117, 120, 122].

5. Mechanisms Underlying HDACI-Mediated Repression of HIF-α Transactivation Potential

Early report suggested that TSA repressed angiogenesis by regulating VHL and p53 function, hence destabilizing HIF-1α [118]. Later observations show that HDACIs also repress
the TAP of the carboxyl-transactivation domain (CAD) of both HIF-1α and HIF-2α [17]. This effect can be clearly demonstrated by using a recombinant HIF-αCAD construct fused to the DNA binding domain of the yeast GAL4 transcription factor. The protein levels of this fusion protein are not decreased by HDACIs, allowing the examination of its activity by monitoring the expression of a reporter gene [17]. All other transactivators tested in the same way, including p300, VP16, MyoD, and p53, were enhanced by HDACIs under the same conditions. The effects of HDACI on the transactivation potential have two special features that are distinct from the destabilizing effects. First, low doses of HDACIs that were not sufficient to cause HIF-1α degradation were sufficient to repress HIF-1α transactivation potential under both normoxic and hypoxic conditions [17]. Second, while HDACIs repress the transactivation potential of both HIF-1α and HIF-2α, they only trigger the destabilization of HIF-1α, not HIF-2α [17, 18]. Because of these two features, this mechanism may be more relevant to the antitumor effects of HDACIs than the HIF-1α destabilization caused by high doses of HDACIs, because it is easier and more practical to achieve a low therapeutic dose in a clinical setting. Scientifically, this is also interesting because it shows the uniqueness of HIF-α among other transcription factors.

It has been well established that HIF function is determined by the protein levels and the transactivation activity of HIF-α. HIF-α has two transactivation domains, the NAD and the CAD. The transactivation activity of CAD is absolutely dependent on the interaction of the CAD with either p300 or CBP. The interaction between HIF-1α and p300 (or CBP) requires an intact CH1 domain of p300 (or CBP). In addition, HIF-1α has been reported to possess a p300/CBP CH1-independent transactivation activity which is also sensitive to HDACIs [129, 130]. Because HIF-αCAD has been demonstrated to be absolutely dependent on p300/CBP CH1 [129], the p300/CBP CH1-independent mechanism might involve the NAD of HIF-α. These reports indirectly indicate that inhibitors of class I/II HDACs also repress the transactivation activity of HIF-αNAD.

Because HDACIs mediate repression of HIF function in a manner independent of HIF-α levels, the key targets of this repression must be the HIF-α-p300 or HIF-α-CBP complexes (Figure 3). In oxygen-sensing pathway, oxygen availability regulates this interaction through FIH (Factor inhibiting HIF-1)-mediated hydroxylation of HIF-αCAD. However, mutation of Asn803 of HIF-1αCAD did not abolish HDACI-mediated repression [17], indicating that the HDACI-mediated repression of HIF-1α-p300 function is independent of either FIH or hydroxylation. The HDACI-mediated repression of HIF-α TAP is also independent of VHL [17], suggesting a mechanism distinct from the normoxic repressive pathway. Since a minimal CAD domain (HIF-1α786-826) lacking the normoxic repressive region thus being constitutively active can be repressed by HDACIs [17], it is unlikely that the HDACI-mediated repression of HIF-αCAD involves a direct change of acetylation states of HIF-α [17], HIF-αNAD, on the other hand, overlaps with the oxygen-dependent degradation domain and contains more than one lysyl residues. So it is possible that acetylation of any of the lysyl residues affects NAD transactivation activity.

While direct acetylation of HIF-α, if any, is unlikely to be involved in HDACI-mediated repression of HIF function, the direct acetylation of p300/CBP, the other determinant of the transactivation activity of HIF complexes, has been well documented. p300 and CBP are acetyltransferases serving as general cofactors for multiple transcription factors including HIF-α [131]. These two proteins possess multiple domains that function as docking sites for their interaction with a variety of transcription regulators [131]. Interestingly, all those important functional domains are lysine-rich and have shown to be subjective to autoacetylation by p300 or CBP [131, 132]. Importantly, exposure of cells to HDACIs causes hyperacetylation of p300 [131]. Consistent with these observations, p300 has been reported to complex with HDAC activities [133–135]. These observations suggest that HDACI-mediated repression of HIF transactivation more likely implicates the acetylation status of p300 or CBP. A recent work revealed that the transactivation activity of HIF-αNAD also requires an interaction with p300 or CBP [136]. This interaction is mediated by CH3 domain, which is also one of the lysine-rich regions subjective to acetylation [131]. Therefore, it is possible that the HDACI-mediated repression of HIF-αNAD also involves the acetylation status of p300 or CBP. Considering that both CH1 and CH3 domains of p300 or CBP are lysine-rich and subjective to acetylation [131] and p300 or CBP physically interacts with deacetylase activity [134], one intriguing hypothesis would be that the acetylation status of CH1 and CH3 may affect their binding affinity to different transcription factors [137]. If it is true, acetylation of p300 and CBP may represent an additional mechanism for these two general coactivators to dynamically coordinate the transcriptional reprogramming of multiple genes. Finally, since multiple signaling pathways regulate HIF-α-p300 complex, it is also possible that one or more signaling pathways are relayed by HDAC activity, or some regulators of the signaling pathways are subjective to acetylation (Figure 3).

6. Mechanisms Underlying HDACI-Mediated Degradation of HIF-1α

As histone acetylation is generally associated with enhanced gene transcription, it is common to find that HDACI enhances the transcription and de novo synthesis of proteins. It is also true in most exogenous gene expression systems including transfection of cultured cells and in vivo gene therapy. The transcription of endogenous HIF-1α, however, is not affected by HDACIs (Chen & Sang, unpublished data). Previous studies from our laboratories and others have shown that HDACI treatment has little effect on the de novo translation of endogenous HIF-1α protein [137]. Here we focus our discussion on HDACI-mediated degradation of HIF-1α.

6.1. Do Inhibitors of Class I/II HDACs Directly Enhance the Acetylation of HIF-1α at Lys532? Interaction between protein acetylation and ubiquitination has been discussed in two
catalytic activity as an α may aggregate human ARD1 [144]. An alternative possibility is that hARD1 with siRNA a role of hARD1 in HIF-1 is implicated in the regulation of cell proliferation, a precise HIF-1 analyzed N-ε-acetylation of HIF-1[140]. Subsequent evidence has shown that hARD1 cannot acetylate human HIF-1α in vitro [140–143]. One explanation for this discrepancy is that mARD1225 has a C-terminal region that significantly differs from those of other mouse or human ARD1 [144]. An alternative possibility is that hARD1 may aggregate in vitro, and aggregated hARD1 loses its catalytic activity as an α-acetylase [145]. Silencing of hARD1 with siRNA affected cell proliferation, but showed no effect on HIF-1α stability [141, 142]. The role of hARD1 in cell proliferation was further demonstrated in mouse xenograft tumor model [146]. Therefore, while published data suggest that mARD1 225 has a role in HIF-1 in vitro [140–143]. One explanation for this discrepancy is that mARD1225 has a role in HIF-1α stability, and hARD1 is implicated in the regulation of cell proliferation, a precise role of hARD1 in HIF-1α stability remains unclear.

HIF-1α is easily detectable from the immunoprecipitates by using anti-acetyl-lysine antibodies [116, 124, 147, 148]. It is also possible that HIF-1α interacts with one or more acetylated proteins, thus is indirectly coprecipitated by antily-sine antibody in immunoprecipitation experiments. More recently, several studies showed direct detection of HIF-1α in immunoblotting with anti-acetyl-lysine antibodies [149]. The involvement of Sirt1, a member of class III deacetylase, in the regulation of HIF-2α has been reported [150]. These reports generally support that HIF-1α may undergo direct acetylation in cells. Nevertheless, a specific role of Lys532 in HDACI-triggered HIF-1α degradation remains unclear. ODD is sufficient to mediate the HDACI-triggered HIF-1α instability; however, mutation of the putative acetylation site (Lys532 to Arg) failed to protect ODD from HDACI-induced degradation [18]. So far there is no direct evidence to support that HDACIs of class I/II enhance HIF-1α acetylation at Lys532 in cells. Mass-Spectrometry analysis of HIF-1α isolated from cells may eventually resolve the acetylation status of Lys532 of HIF-1α and shed light on its role in HIF-1α stability.

6.2. HDACIs Induce Ubiquitination-Independent Degradation of HIF-1α. In the original model proposed by Dr. Kim, HIF-1α acetylation at Lys532, either catalyzed by mARD1 or induced by HDACIs, promotes HIF-1α recognition and eventual ubiquitination by VHL [118, 124]. Since HDACIs enhance the interaction between HIF-1α and HSP70, an alternative ubiquitination pathway mediated by HSP70-associated CHIP has been proposed [151]. Ubiquitination of proteins sequentially involves three enzyme activities termed E1, E2, and E3. Mammalian cells have a single ubiquitin-activating enzyme E1, and VHL is the HIF-α-specific E3 ligase. Accordingly, VHL-defective cells or E1-inactivated cells accumulate high levels of HIF-α. If HDACI-triggered HIF-1α degradation was mediated by the canonical ubiquitination pathway, the process would depend on functional E1 and VHL activity. In fact, it is reported that HDACIs decreased HIF-1α levels in all cells tested, including VHL (−/−) C2 and RCC4 cells, indicating that HDACI-induced HIF-1α degradation is through a mechanism existing in, perhaps, all tumors, including those lacking VHL. A special cell line, Ts20TGR, contains a temperature sensitive E1 [152]. Inactivating E1 in this cell line by culturing the cells at 39°C resulted in accumulation of nonubiquitinated HIF-1α [18]. Apparently, HDACIs are able to trigger degradation of the accumulated nonubiquitinated HIF-1α. Since the HDACI-triggered degradation can be blocked by proteasome inhibitors, but not by lysosomal inhibitors, it is clear that the proteasome system is required [18]. Based on the above facts, it is clear that HDACIs induce HIF-1α destruction by a ubiquitination-independent proteasome system (UIPS), whereas the precise mechanism remains to be dissected.

6.3. Is Hsp90 the Major Player in HDACI-Triggered Degradation of HIF-1α? Studies with Hsp90 inhibitors reveal a good candidate which may be responsible for HDAC-triggered degradation of HIF-1α. Hsp90 is known to associate with nonnative structures of many proteins and is responsible for protein folding in general [153]. Hsp90 have three functional domains, the ATP binding domain, protein binding domain, and dimerization domain. The normal function of Hsp90 depends on its ATPase activity because it is the principal binding site for drugs that target this protein [154]. Hsp90 inhibitors have also been explored as antitumor drugs [155]. A quick comparison reveals some obvious similarities between these two groups of drugs. (1) Both HDACI and Hsp90 inhibitors have been reported to destabilize various mutated HSP90 client proteins in cells; (2) both groups of drugs enhance the levels of HSP70; (3) both groups of drugs decrease client proteins’ interaction with HSP90 but increase its interaction with HSP70; (4) while HDACIs apparently inhibit deacetylases, since HSP90 function requires HDAC6 activity to maintain its deacetylated states [156–160], HDACIs function as HSP90 inhibitors indirectly (Figure 4).

It has been reported by independent laboratories that molecular chaperones including HSP70 and HSP90 directly interact with HIF-1α, suggesting that HIF-1α is one of the client proteins of the HSP machinery. Similar to HDACIs, the Hsp90 inhibitor 17-AGG triggers ubiquitination independent degradation of HIF-1α [17, 18]. We noted that most of the reported protein degradation cases triggered by either HDACI or Hsp90 inhibitor were observed in cells with normal ubiquitination system. So even though the proteins subjective to the drug-induced degradation were generally associated with ubiquitination, there is no real evidence to support that ubiquitination is an absolute prerequisite for their degradation.

6.4. Potential Role of α-Tubulin Acetylation in HDACIs-Mediated Degradation of HIF-1α. Another possible acetylated protein that may play a role in HDACI-induced destabilization of HIF-1α is the α subunit of tubulin heterodimers (α-tubulin). α-tubulin is an important component for the
formation of microtubules and other cellular structures with a variety of functions. The acetylation of α-tubulin was discovered long before the identification of histone deacetylase and protein acetyl transferases [161]. Its acetylation at Lys40 is a marker of stabilized microtubules [41, 43, 162] and is regulated by HDAC6 [163]. In addition, HDAC6 provides a link between protein acetylation and ubiquitination [164], suggesting a role in regulating protein stability. Since a link between protein acetylation and ubiquitination [164], [18, 41, 137], one possibility is that microtubule dynamics may somehow be required for HIF-1α stabilization. This hypothesis is supported by evidence that the small molecules disrupting the dynamics of microtubules also destabilize HIF1α [113, 114, 165]. However, how and why microtubule dynamics affects HIF-1α stability remains unknown.

7. The Acetylases and Deacetylases Involved in HIF Function

Because of the complexity and possible functional redundancy, it may be difficult to identify an individual member that is exclusively responsible for the regulation of HIF acetylation and function. As discussed above, the role of hARD1 in HIF-1α acetylation is controversial. A role of HDAC7 in regulating HIF-1 protein stability was first proposed, based on its interaction with HIF-1α but not HIF-2α [117]. HDAC7 was found to increase the transactivation activity of HIF-1, and it is thought to be a transactivation coactivator of HIF-1 [117]. So far several Class II HDACs have been proposed to regulate HIF-1α stability [116]. However, since HDAC7 does not interact with HIF-2α, it cannot be used to fully explain the repressive effects of HDACIs on HIF-2αCAD. It is shown that HDAC4 and HDAC6 coimmunoprecipitated with HIF-1α and the specific inhibition of HDAC4 and HDAC6 repress HIF-1α stability [116]. It is possible that multiple deacetylases are involved in HDACI-induced modulation of HIF function, and that different cell types, different physiological conditions or signaling pathways may implicate different HDACs in the regulation of HIF function.

8. Conclusions and Perspective

The above discussion is based on experimental evidence and published literature that may link the biochemical effects of HDACIs to the repression of HIF function. The discussions are generally focused on deacetylases, acetylation substrates, and their potential relevance to the regulation of HIF function. It is clear that the transcription complexes of HIF-1 and HIF-2 require an activity of type I/II deacetylase for their transactivation activity. This deacetylase-dependent transactivation represents a unique feature of HIF function. It is also conclusive that higher doses of HDACIs induce the degradation of HIF-1α through a proteasome-dependent pathway. This degradation can be mediated by an ubiquitination-independent mechanism. We expect further investigation in this field would bring new insight into the molecular and biochemical mechanisms underlying the anti-HIF and antiangiogenic effects of inhibitors of type I/II HDACs. It is also important to point out that a member of the class III HDACs, Sirt1, has been reported to deacetylate HIF-1α and HIF-2α and repress HIF-α activity [150, 166], further showing the complexity of effect of acetylation on HIF function. A thorough understanding of the regulation of HIF-α by protein acetylation is essential for future exploration aiming to modulate HIF function in vivo by targeting HDACs.

While it is conclusive that in addition to serving as epigenetic therapeutics, the inhibitors for class I and II deacetylases also repress HIF function, the underlying mechanisms remain far from clear. A better understanding of the mechanisms may be beneficial not only for better efficacy of cancer therapy, but also for prevention of side effects to normal organs. Particularly, given the large number of deacetylases and their important roles in transcriptional regulation, epigenetic programming, chromosomal remodeling, and other cellular processes, it is possible that nonselectively blocking deacetylases may cause unpredictable side effects. Obviously, a nonexhaustive list of imminent future directions should include (1) identifying the acetylases and deacetylases involved in HIF function under defined conditions in specific cell types, (2) identifying the HIF regulatory
factors subjective to acetylation, (3) defining the specific acetylation sites of their substrates and their relevance to HIF-α, (4) defining the upstream signaling pathways that regulate HIF function through protein acetylation. At least, a recent elegant study has linked cellular metabolic to protein acetylation. Considering that HIF function is required for the maintenance of oxygen and nutrient supply and for prevention of cell death under hypoxic conditions, global repression of HIF-α activity in the entire body, particularly for long-term use, may affect chronic adaptation required for ischemic disorders. On the other hand, since HIF function and disregulated expression of VEGF play roles in tissue damage caused by ischemia-reperfusion, HDACI-mediated repression of HIF may prove to be beneficial for acute ischemia [168]. The effects of HDACIs on endothelial, bone marrow, neuronal, and circulatory systems warrant a thorough interrogation [169, 170]. Taken the potential adverse effects into consideration, a lesion-specific activation of prodrugs, which can be either HDACIs or compounds specifically regulating HIF function, may become an exciting exploration.

Abbreviations

CAD: Carboxyl-terminal transactivation domain
CHIP: Carboxyl terminus of HSP70-interacting protein
FDA: Food and Drug Administration (USA)
FIH: Factor inhibiting HIF-1
HAT: Histone acetyltransferase
HADC: Histone deacetylase
HDACI: Histone deacetylase inhibitor
HIF: Hypoxia-inducible factors
HSP: Heat shock protein
HUPS: Hydroxylation-ubiquitination-proteasomal system
NAD: Amino-terminal transactivation domain
ODD: Oxygen-dependent degradation domain
PHD: Prolyl hydroxylases
TAP: Transactivation potential
UIPS: Ubiquitination independent proteasomal system
VEGF: vascular endothelial growth factor.

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