Class II Histone Deacetylases Are Associated with VHL-Independent Regulation of Hypoxia-Inducible Factor 1α

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Abstract

Hypoxia-inducible factor 1α (HIF-1α) plays a critical role in transcriptional gene activation involved in tumor angiogenesis. A novel class of agents, the histone deacetylase (HDAC) inhibitors, has been shown to inhibit tumor angiogenesis and HIF-1α protein expression. However, the molecular mechanism responsible for this inhibition remains to be elucidated. In the current study, we investigated the molecular link between HIF-1α inhibition and HDAC inhibition. Treatment of the VHL-deficient human renal cell carcinoma cell line UMRC2 with the hydroxamic HDAC inhibitor LAQ824 resulted in a dose-dependent inhibition of HIF-1α protein via a VHL-independent mechanism and reduction of HIF-1α transcriptional activity. HIF-1α inhibition by LAQ824 was associated with HIF-1α acetylation and polyubiquitination. HIF-1α immunoprecipitates contained HDAC activity. Then, we tested different classes of HDAC inhibitors with diverse inhibitory activity of class I versus class II HDACs and assessed their capability of targeting HIF-1α. Hydroxamic acid derivatives with known activity against both class I and class II HDACs were effective in inhibiting HIF-1α at low nanomolar concentrations. In contrast, valproic acid and trapoxin were able to inhibit HIF-1α only at concentrations that are effective against class II HDACs. Coimmunoprecipitation studies showed that class II HDAC4 and HDAC6 were associated with HIF-1α protein. Inhibition by small interfering RNA of HDAC4 and HDAC6 reduced HIF-1α protein expression and transcriptional activity. Taken together, these results suggest that class II HDACs are associated with HIF-1α stability and provide a rationale for targeting HIF-1α with HDAC inhibitors against class II isozymes. (Cancer Res 2006; 66(17): 8814-21)

Introduction

Hypoxia-inducible factor 1α (HIF-1α) is an important transcription factor regulating gene expression in erythropoiesis, angiogenesis, and glycolytic metabolism (1). Under normoxic conditions, HIF-1α is hydroxylated by prolyl hydroxylase domain oxygenases. The hydroxylated proline serves as a recognition signal for E3 ubiquitin ligase VHL complex binding and subsequent targeting for polyubiquitination and proteasomal degradation. Under hypoxic conditions, the oxygen-dependent prolyl hydroxylase domains are not active and HIF-1α is not hydroxylated for VHL binding and degradation. Stable HIF-1α translocates into the nucleus, dimerizes with HIF-1β, and activates the transcription of target genes.

During cancer progression, inefficient tumor vasculature hampers oxygen delivery and produces intermittent hypoxia. As a result, hypoxic tumor cells accumulate HIF-1α protein, which in turn transactivates genes promoting adaptation, survival, angiogenesis, and metastases. In several human tumor types, HIF-1α can also be stabilized under normoxic conditions due to the abnormal oncogenic signaling pathways (1). More than 50% of renal cell carcinomas have von Hipple-Lindau (VHL) gene inactivation by deletion, mutation or an epigenetic mechanism (2). HIF-1α is constitutively accumulated in renal cell carcinoma both in cell lines and in tumors.

Reintroduction of wild-type VHL can significantly reduce the HIF-1α protein level and impair tumor growth *in vivo* (2, 3). In addition, targeting the chaperone function of heat shock protein 90 (Hsp90; i.e., geldanamycin) can trigger a VHL-independent HIF-1α degradation pathway under both normoxic and hypoxic conditions (4). In addition to proline hydroxylation, HIF-1α can also be acetylated at Lys^{532} by the acetyltransferase ARD1 (5). The acetylation promotes HIF-1α interaction with VHL and subsequent degradation.

Histone acetylation is important for DNA chromatin structure and gene transcription regulation. Nonhistone protein acetylation has been shown to regulate protein function and stability. The reversible acetylation of histone and nonhistone proteins at the lysine residue is controlled by histone deacetylases (HDAC) and histone acetyltransferases (6–8). HDAC isozymes can be categorized into three classes: class I includes HDAC1, HDAC2, HDAC3, and HDAC8. All class I HDACs are localized in the nucleus and act as transcriptional corepressors by deacetylation of chromatin histone proteins and other DNA binding proteins. Class II includes HDAC4, HDAC5, HDAC6, HDAC7, HDAC9, and HDAC10. Most of them can be regulated and shuttled between the cytoplasm and the nucleus in response to various signal transduction stimuli. In addition, class II HDACs exert their transcriptional corepressor functions by interacting with (or deacetylating) other corepressors or direct binding to (and sequestering) sequence-specific transcriptional factors such as MEF2, Runx3, and nuclear factor κB (NF-κB; refs. 8, 9). Class III HDACs are Sir2 family deacetylases, which deacetylate many nonhistone proteins but need NAD for their activities (8).

HDAC inhibitors are a new class of anticancer agents designed to inhibit HDACs and to allow histone acetyltransferases to induce histone acetylation and, consequently, gene transcription by opening the chromatin structure. Treatment of transformed cells with HDAC inhibitors results in growth inhibition (10). This phenotype can be due to gene transcriptional activation and/or other unknown mechanisms related to HDAC inhibition. HDAC

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inhibitors are a group of structurally diverse small-molecule compounds. They can be categorized into four groups: hydroxamic acids, cyclic peptides, aliphatic acids, and benzamides. None of the HDAC inhibitors currently in clinical development can specifically inhibit a single HDAC isozyme. Hydroxamic acid derivative HDAC inhibitors such as trichostatin A, suberoylanilide hydroxamic acid, LAQ824, and LBH589 are pan-HDAC inhibitors that target both class I and class II HDACs. Cyclic peptides, aliphatic acids, and benzamides have inhibitory activity against class I HDACs but have limited activity against class II HDACs at higher concentrations (11–14). HDAC inhibitors such as trichostatin A, LAQ824, FK228, and sodium butyrate have all been reported to inhibit HIF-1α in cancer cell lines (5, 15, 16).

In our previous studies, we used hydroxamic-based HDAC inhibitors to target HIF-1α in prostate and breast cancer cell lines (15). Plasma achievable concentrations of LAQ824 did not change the HIF-1α mRNA level but significantly reduced HIF-1α protein under both normoxic and hypoxic conditions. Furthermore, HIF-1α inhibition correlated with downstream vascular endothelial growth factor (VEGF) inhibition. One possible mechanism may be due to inhibition of HDACs and increase of HIF-1α protein acetylation by ARD1, and subsequent VHL-dependent targeting for degradation (5). However, the role of ARD1 in HIF-1α acetylation has been recently disputed (17). The molecular link between HIF-1α inhibition and HDAC inhibition has yet to be fully established.

In the current study, we used a VHL-deficient renal cell carcinoma cell line and investigated the posttranscriptional modulation of HIF-1α by HDAC inhibitors. By testing structurally different HDAC inhibitors, we determined that effective HIF-1α inhibition requires class II HDAC inhibition. Our results showed that HDAC4 and HDAC6 coimmunoprecipitate with HIF-1α, and specific inhibition of HDAC4 and HDAC6 compromised HIF-1α stability and transcriptional activity.

Materials and Methods

Cell culture and HDAC inhibitors. VHL-deficient renal cell carcinoma cell line UMRC2 (C2) and C2 cells with permanent wild-type VHL transfection (C2VHL; ref. 18) were kindly provided by Drs. Jennifer Isaacs and Len Neckers (National Cancer Institute, NIH, Bethesda, MD). Cells were cultured in DMEM medium with 10% fetal bovine serum at 37°C in a 5% CO_2 and 95% air incubator. The hypoxic accumulation of HIF-1α was induced by 100 μmol/L $CoCl_2$ (Sigma, St. Louis, MO). HDAC inhibitors LAQ824, LBH589, and trapoxin were provided by Novartis (Cambridge, MA). Valproic acid was purchased from Sigma. LAQ824 and LBH589 were dissolved in DMSO as 10 mmol/L stocks and diluted with cell culture medium before experiments. Valproic acid was dissolved in water solution before experiments.

Western blot analysis. Western blot analyses were carried out on either 4% to 15% gradient gel or 7.5% gel according to methods previously described (15). The monoclonal or polyclonal antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA; HDAC4, HDAC6), Cell Signaling (Beverly, MA; acetyl-lysine, ubiquitin 1, Hsp90, Hsp70), and R&D Systems (Minneapolis, MN; HIF-1α).

Immunoprecipitation experiments and HDAC activity assay. For immunoprecipitation of endogenous proteins, 10×10^6 cells were lysed and subjected to immunoprecipitation with antibodies specific for HDAC4, HDAC6 (Santa Cruz Biotechnology), HIF-1α (R&D System), and acetyl-lysine (Cell Signaling) at 2 μg per reaction using protein A/G agarose (Sigma). The immunocomplex was subjected to Westerns blot analysis or HDAC activity assay using a nonradioactive HDAC assay kit based on the instructions by the manufacturer (Biomol International, Plymouth Meeting, PA). Briefly, the agarose beads containing HIF-1α immunocomplex were incubated with 150 μL of 100 mmol/L acetylated substrates for a maximum of 45 minutes with gentle rocking. As negative controls, the same amounts of agarose beads without going through immunoprecipitation assays were incubated with the substrates. After incubation, equal aliquots from each condition were mixed with developer solution, and fluorescence intensity was read at excitation 360 nm and emission 460 nm. The fluorescence intensity from each condition was adjusted with the negative controls and normalized to immunoglobulin G (IgG).

Reporter gene assay. The hypoxia response element (HRE) driving firefly luciferase expression plasmid (p2.1) and the control plasmid (pTK-Renilla) were a kind gift from Dr. Gregg Semenza (Johns Hopkins School of Medicine, Baltimore, MD), and characterized previously (19, 20). C2 cells were seeded into 12-well plates and allowed to reach ~50% confluence. Then, different doses of LAQ824 were added into the culture. After overnight treatment, cells were cotransfected with 1 μg of p2.1 and 0.1 μg of pTK-Renilla using Fugene6 (Roche, Indianapolis, IN). After 24 hours, the cell lysates were analyzed with a Dual Luciferase Reporter Assay kit (Promega, Madison, WI). The relative luciferase activity was determined by the ratio of firefly to Renilla luciferase activity.

Short hairpin RNA assay. To knock out HDAC4 and HDAC6, a short hairpin RNA (shRNA) was designed. Briefly, shRNAs were designed to target the nucleotide 30-60, 444-474, and 1,484-1,514 regions of the HDAC4 coding sequence. For HDAC6 shRNA, coding sequences of nucleotides 179-203, 271-295, and 423-447 were initially targeted. The shRNA-encoding complementary single-stranded oligonucleotides, which hybridize to give *Bse*RI- and *Bam*HI-compatible overhangs, were designed for ligation to pSHAG1 vector (kindly provided by Dr. Greg Hannon, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY). The shRNA that gave the maximum knockdown (nucleotides 444-474 for HDAC4 and nucleotides 271-295 for HDAC6) was selected for further experiments. The transfection was carried out in six-well plates on cells with 50% confluence using Lipofectamine 2000 (Invitrogen, Carlsbad, CA). The transfection was repeated after 24 hours. Seventy-two hours after the initial transfection, cells were lysed for protein and Western blot analysis. For the functional assay, 24 to 48 hours after the second transfection, cells were cotransfected with p2.1 plasmid and pTK-Renilla plasmid. Dual luciferase reporter assays were done 24 hours later.

Statistical analysis. Differences between the means of unpaired samples were evaluated by the Student's t test using the SigmaPlot and SigmaStats program. $P < 0.05$ was considered statistically significant. All statistical tests were two sided.

Results

The hydroxamic acid derivative LAQ824 induces HIF-1α degradation and transcriptional inactivation via a VHL-independent mechanism. To investigate the molecular mechanism underlying HDAC inhibitor mediated HIF-1α inhibition, we used a human renal cell carcinoma cell line UMRC2 (C2) with no functional VHL, and a C2 cell line permanently transfected with wild-type VHL gene—UMRC2VHL (C2VHL). Western blot analysis revealed a significant HIF-1α protein accumulation in C2 cells under normoxic conditions (Fig. 1*A*). The presence of wild-type VHL prevented HIF-1α accumulation under normoxic conditions but not following treatment with the hypoxic mimic cobalt chloride. LAQ824 effectively inhibited HIF-1α protein accumulation in both C2 and C2VHL cells starting at 0.05 μmol/L with no significant change in HIF-1α mRNA levels (Fig. 1*A*). The dose-dependent inhibition of HIF-1α by LAQ824 was associated with the inhibition of HDAC activity as shown by the induction of p21 protein expression and an increase of both histone (H3) and nonhistone protein (tubulin) acetylation (Fig. 1*B*).

To further show the functional consequence of HIF-1α inhibition, we pretreated C2 cells with increasing doses of LAQ824 for 24 hours, and then transiently transfected them with

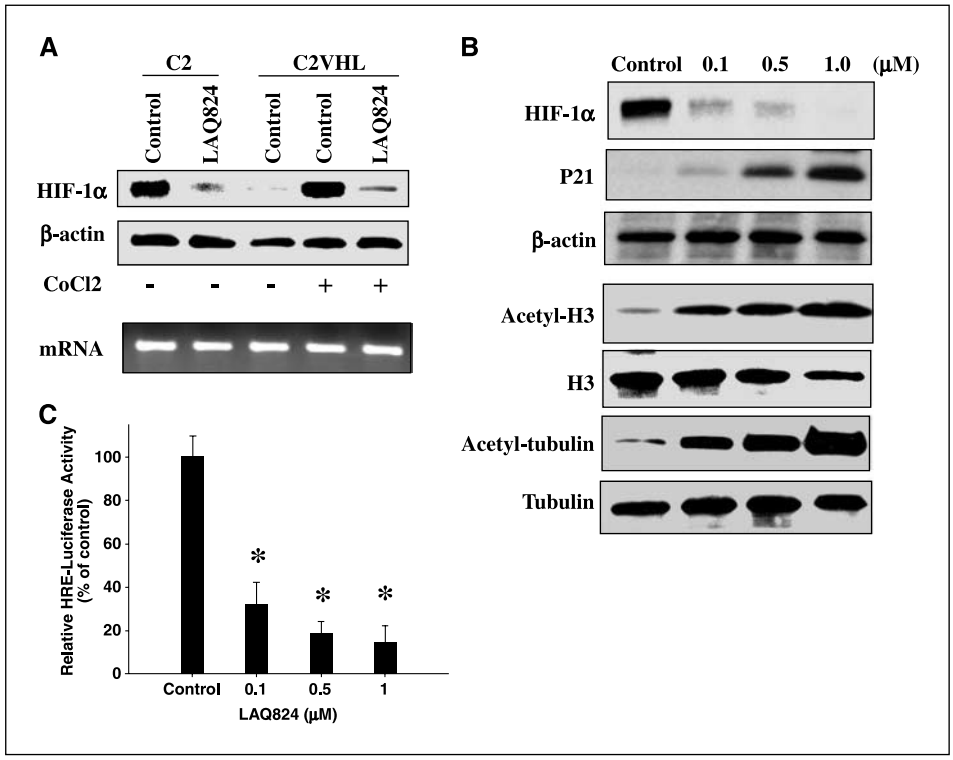

Figure 1. VHL-independent inhibition of HIF-1α by LAQ824. *A,* C2 and C2VHL cells were treated with 50 nmol/L LAQ824 for 24 hours. To mimic hypoxia, 100 μmol/L $CoCl_2$ was added to the culture medium for the last 6 hours of treatment in the C2VHL cultures. For each condition, 50 μg of total protein lysates were used for Western blot analysis to detect HIF-1α protein levels. The same blot was probed with β-actin as equal loading control. For HIF-1α mRNA analysis, similar cell culture experiments were done, followed by reverse transcription-PCR using primers specific for HIF-1α. *B,* the dose-dependent effect of LAQ824 on HIF-1α inhibition was associated HDAC inhibitory properties. Cells were treated for 24 hours and 50 μg of total proteins were used for Western blot analysis. *C,* HIF-1α protein reduction resulted in diminished transcriptional activity. C2 cells were treated with different doses of LAQ824 as in (*B*). The cells were then cotransfected with HRE-firefly luciferase vector (p2.1) and pTK-Renilla vector as the control. Twenty-four hours after transfection, dual luciferase activity was measured and the ratio of firefly/Renilla was used as the relative luciferase activity. The relative luciferase activity from samples treated with solvent controls was considered as 100%. *Columns,* mean from three independent experiments; *bars,* SD. *, $P < 0.05$, versus control.

a hypoxic responsive element (HRE)–driven firefly luciferase reporter gene vector (p2.1), of which expression is based on the availability of HIF-1α. As a transfection control, a TK-driven Renilla luciferase vector was also cotransfected. Following a 24-hour cotransfection, C2 cells were assayed for luciferase activity. The inhibition of HIF-1α protein resulted into a dose-dependent reduction of p2.1 expression and HRE-driven luciferase activity (Fig. 1*C*).

LAQ824-induced HIF-1α inhibition is associated with protein acetylation and ubiquitination. We have previously shown in prostate and breast cancer cells that inhibition of HIF-1α by LAQ824 did not occur at the gene transcription level (15). Treatment with 0.1 μmol/L LAQ824 ablated HIF-1α protein expression in C2 cells (Fig. 2*A*). However, cotreatment of C2 cells with proteasomal inhibitor MG132 rescued HIF-1α protein from degradation induced by LAQ824. These results indicate that LAQ824 induces HIF-1α protein degradation independently of functional VHL protein in renal cell carcinoma cells (Figs. 1 and 2*A*). Following 24-hour treatment with LAQ824 in the presence of the proteasomal inhibitor MG132 during the last 12 hours of incubation, we assessed HIF-1α ubiquitination. Protein immunoprecipitation with monoclonal anti-HIF-1α antibody followed by immunoblotting with a monoclonal antibody against ubiquitin revealed that HIF-1α protein was also ubiquitinated (Fig. 2*B*). The bands at 250 and 150 kDa represent polyubiquitinated and monoubiquitinated HIF-1α, respectively. These data suggest that LAQ824-induced HIF-1α degradation is also associated with ubiquitination despite the absence of functional VHL protein. In a recent paper, VHL wild-type cancer cells treated with trichostatin A induced HIF-1α degradation via protein acetylation and VHL-mediated ubiquitination (5). In our report, LAQ824-induced HIF-1α acetylation preceded HIF-1α protein degradation in VHL-negative C2 cells. As early as 4 hours after the treatment with LAQ824, the protein immunoprecipitation experiment indicated a dose-dependent increase of the HIF-1α protein acetylation level (Fig. 2*C*). At this time point, LAQ824 did not induce any significant decrease of HIF-1α protein. Indeed, HIF-1α degradation did not occur until 12 hours after LAQ824 exposure (data not shown). HIF-1α protein stability can also be affected by the heat shock proteins Hsp90 and Hsp70. Because HDAC inhibitors have been reported to induce Hsp90 acetylation and cause the disassociation of client proteins, we also investigated the association of HIF-1α with Hsp90 and Hsp70 in our model. Following 4 and 8 hours of treatment with 0.5 μmol/L LAQ824, there were no significant changes in HIF-1α/Hsp90 and HIF-1α/Hsp70 association as indicated by coimmunoprecipitation experiments (Fig. 2*D*). Interestingly, 4-hour treatment with LBH-589 induced a significant down-regulation of HIF-1/HRE activity in C2 cells transiently transfected with HRE-driving luciferase reporter gene (Fig. 2*E*). These results suggest that HIF-1α acetylation may also compromise HIF-1α transcriptional activity.

HIF-1α immunocomplex contains HDAC activity. To further characterize the molecular mechanisms underlying LAQ824-induced HIF-1α inhibition, we did protein immunoprecipitation using a monoclonal antibody against HIF-1α in the protein lysates from C2 cells. The protein A/G agarose gels containing the immunocomplex for HIF-1α were pooled and used in the HDAC activity assay. HDAC activity was detected in the immunocomplex precipitated using HIF-1α antibody compared with the IgG controls. More importantly, HDAC activity was inhibited during the assay in the presence of 0.5 μmol/L LAQ824 (Fig. 3). These data suggest a molecular link between HDAC inhibition and HIF-1α inhibition. The direct association between HIF-1α protein and HDAC activity provides the rationale for using HDAC inhibitors targeting HIF-1α stability.

Structurally different HDAC inhibitors and HIF-1α inhibition. Different HDAC inhibitors (i.e., trichostatin A, FK228, and sodium butyrate) have been reported to have HIF-1α inhibitory activity (5, 15, 16). To investigate the effect of structurally different HDAC inhibitors on HIF-1α stability, we treated C2 cells with valproic acid, an aliphatic acid–based HDAC inhibitor that is structurally different from hydroxamic acid. As expected, after 24 hours of treatment, 1 mmol/L valproic acid treatment effectively induced histone H3 acetylation and p21 expression, which have been reported to be regulated in the nucleus by class I HDACs (Fig. 4*A*). In contrast, HIF-1α inhibition only occurred at concentrations ≥2 mmol/L. A similar trend was also observed in VHL functional cells. C2VHL cells did not have HIF-1α accumulation until the induction by the hypoxic mimic $CoCl_2$. Pretreatment with high concentrations of valproic acid (≥2 mmol/L) inhibited HIF-1α accumulation whereas a low nanomolar

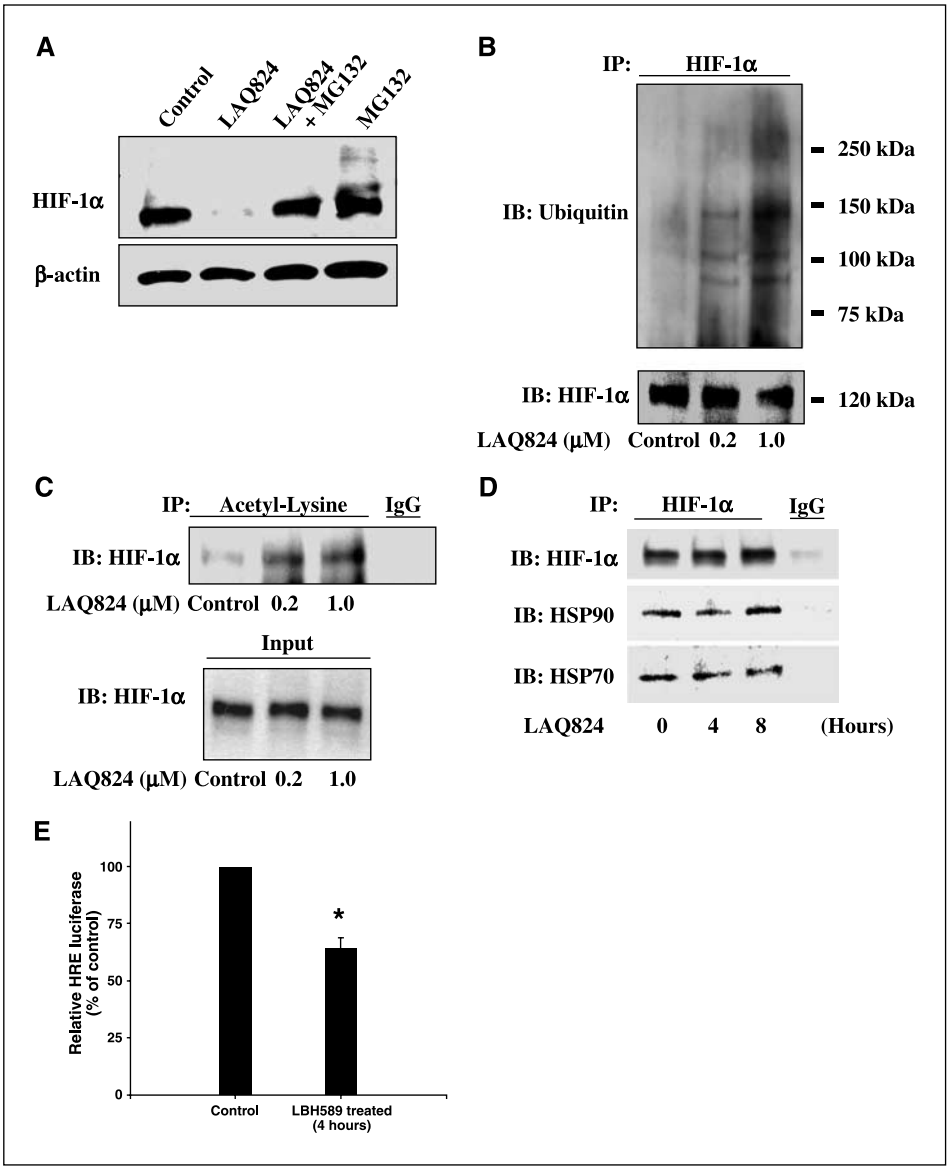

Figure 2. LAQ824-induced HIF-1α protein inhibition is associated with acetylation and polyubiquitination. *A,* proteasome inhibition rescued HIF-1α from LAQ824-induced inhibition. C2 cells were treated with 100 nmol/L LAQ824 for 24 hours. Proteasome inhibitor MG132 was added to the cultures 12 hours before protein harvest either alone or in combination with LAQ824. Fifty micrograms of whole-cell lysates were loaded for each lane. *B,* HIF-1α degradation in VHL-deficient C2 cells involves polyubiquitination. C2 cells were treated with 0.2 and 1.0 μmol/L LAQ824 for 24 hours with the final 12 hours in the presence of MG132. Total proteins were harvested for immunoprecipitation assays with a monoclonal antibody against HIF-1α. Immunocomplexes were analyzed by Western blot analysis with antibodies against ubiquitin and HIF-1α. *C,* LAQ824 induces HIF-1α acetylation. C2 cells were treated with LAQ824 for 4 hours. Then, whole-cell protein lysates were harvested from 10^7 cells in each condition. Five percent of the proteins were saved as input. The remaining 95% was adjusted to equal concentrations with lysis buffer and subjected to immunoprecipitation assays with a rabbit antibody against acetyl-lysine at 2 μg per reaction (2 μg of rabbit IgG was used as negative control). Immunocomplexes were subject to Western blot analysis for HIF-1α. Fifty micrograms of total protein lysates from each condition were used for Western blot as input controls. *D,* LAQ824 did not affect the association between HIF-1α and Hsp90/Hsp70 at early time points. C2 cells were treated with 0.5 μmol/L LAQ824 for 0, 4, and 8 hours. Whole-cell protein lysates were harvested and 1 mg of proteins for each condition was used for immunoprecipitation experiments using antibody specific for HIF-1α. After stringent washes, proteins were eluded with loading buffer and Western blot analysis of HIF-1α, Hsp90, and Hsp70 was done using specific antibodies. *E,* 4-hour treatment with LAQ824 decreased HIF-1α transactivation ability. C2 cells were transiently cotransfected with reporter gene vector for HRE-driving firefly luciferase and TK Renilla luciferase vector for 24 hours. Then cells were treated with 0.5 μmol/L LAQ824 for 4 hours. Dual luciferase activities were measured as described in Fig. 1*C*. *, $P < 0.05$, versus control (Student's *t* test).

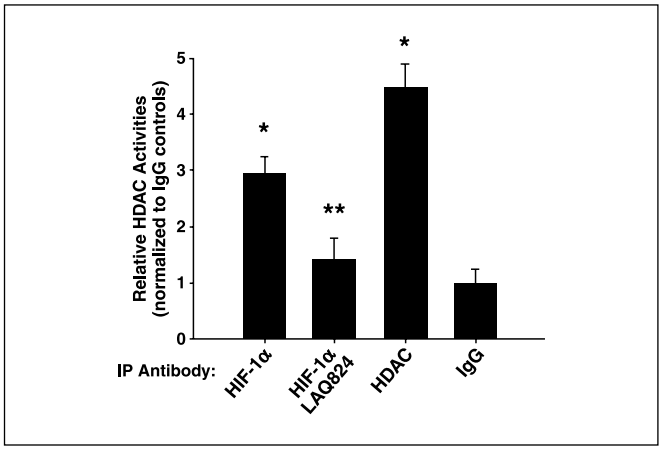

Figure 3. HIF-1α immunocomplex has HDAC activity. Whole-cell protein lysates from C2 cells were immunoprecipitated with either anti-HIF-1α antibody, anti-HDAC6 antibody, or mouse IgG as control (2 μg per reaction). Fluorometric HDAC activity assay was done. The adjusted fluorescence readings were normalized to IgG. *Columns,* mean of three immunoprecipitation and activity experiments; *bars,* SD. *, $P < 0.05$, versus mouse IgG control immunoprecipitation; **, $P < 0.05$, versus HIF-1α immunoprecipitation.

concentration of another hydroxamic acid derivative, LBH589, induced a significant HIF-1α inhibition (Fig. 4*B*). The valproic acid IC_{50} for class I HDACs has been reported to be 0.7 to 1.0 mmol/L whereas the IC_{50} for class II HDACs is ≥1.5 mmol/L (13, 14). Similar to valproic acid, a benzamide derivative HDAC inhibitor only inhibited HIF-1α at concentrations relevant to class II HDAC inhibition (data not shown). Additionally, trapoxin, a cyclic peptide class HDAC inhibitor that is effective in targeting both class I and class II HDACs with the exception of HDAC6 (8), exhibited limited inhibitory activity against HIF-1α at high concentrations (Fig. 4*C*).

Our results suggest that structurally diverse HDAC inhibitors have different inhibitory effects on HIF-1α protein, and the different potency may be due to the effectiveness in targeting one or multiple class II HDACs. The reason for hydroxamic acid derivative HDAC inhibitors such as LAQ824 and LBH589 being more effective is probably due to the fact that they inhibit both class I and class II HDACs at concentrations as low as 25 nmol/L.

Class II HDACs associate with HIF-1α in C2 cells. Class I HDACs (HDAC1, HDAC2, and HDAC3) have been reported to be associated with VHL (21). Based on the data in Fig. 4, we hypothesized that class II HDACs are involved in the regulation of HIF-1α protein degradation in C2 cells. To further investigate the molecular link between HDACs and HIF-1α, we immunoprecipitated whole-cell protein lysates using antibody against HDAC4 and HDAC6 as representative isozymes of class IIa and class IIb HDACs, respectively (Fig. 5*A*). The precipitated protein complexes were subjected to immunoblotting using antibody against HIF-1α. HIF-1α protein was detected in both the HDAC4 and the HDAC6 immunocomplex (Fig. 5*A*).

Next, we carried out RNA interference experiments to deplete the endogenous HDAC4 and HDAC6 in C2 cells. Vector-based shRNA against HDAC4 and HDAC6 did not perturb cell proliferation or viability compared with transfection agents and empty vector containing no shRNA sequence. No changes in the expression of other HDAC isozymes such as HDAC1 and HDAC2 were observed (data not shown). Two consecutive rounds of transfection over a period of 72 hours effectively knocked down ~56% of HDAC4 and 45% of HDAC6 protein expression as compared with negative controls by densitometry analysis of representative Western blots (Fig. 5*B*). More importantly, the HIF-1α protein levels were also reduced (49% with HDAC4 shRNA and 40% with HDAC6 shRNA) as compared with negative controls and endogenous Hsp90 protein expression (Fig. 5*B*). As previously reported, the shRNA inhibition of HDAC6 up-regulated the tubulin acetylation due to the loss of tubulin deacetylase activity associated with HDAC6 (Fig. 5*B*). Then, to assess whether class II HDAC inhibition impairs also the functional activity of HIF-1α, we cotransfected cells with HRE driving firefly luciferase vector (p2.1) and TK-Renilla luciferase vector during the second round of shRNA. Twenty-four hours after the transfection, luciferase assays revealed significant inhibition of HIF-1α/HRE activity (Fig. 5*C*). shRNA against HDAC4 and HDAC6 inhibited 80% and 75% of HIF-1α driving luciferase expression as compared with empty vector controls, respectively. Finally, to investigate whether the

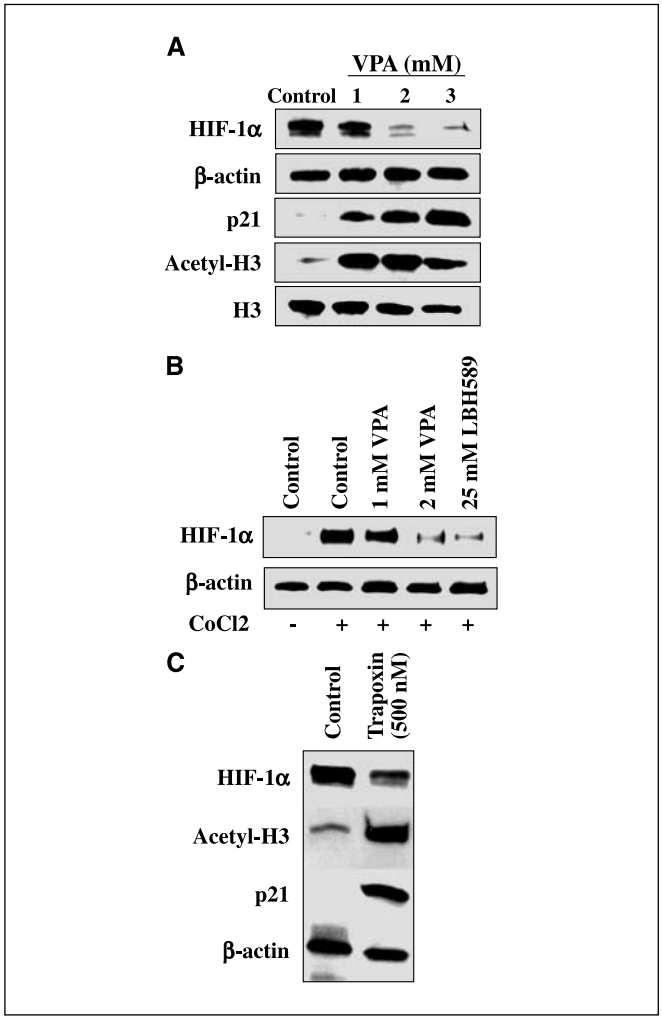

Figure 4. Modulation of HIF-1α by different classes of HDAC inhibitors. *A,* C2 cells were treated with increasing concentrations of valproic acid (*VPA*) for 24 hours and whole-cell lysates were harvested for Western blot analysis for HIF-1α and other markers (p21 expression and histone acetylation) of HDAC inhibition. *B,* C2VHL cells were treated with either valproic acid at the indicated doses or 25 nmol/L LBH589 for 24 hours with the final 6 hours in the presence of $CoCl_2$ for HIF-1α accumulation. Fifty micrograms of whole-cell lysates were used for Western blot analysis. *C,* C2 cells were treated with 500 nmol/L trapoxin for 24 hours and whole-cell lysates were harvested for Western blot analysis for HIF-1α, p21 expression, and H3 histone acetylation.

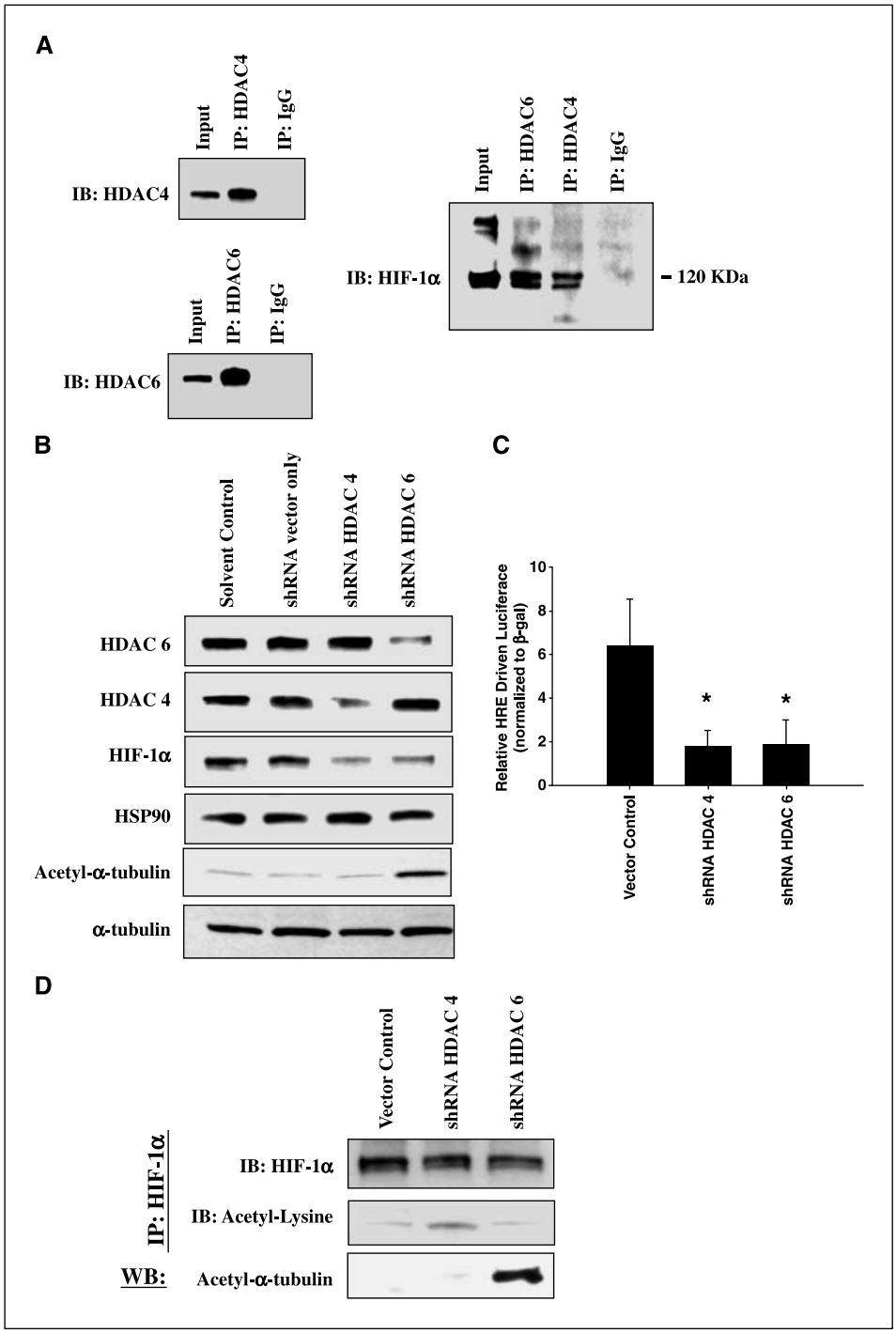

Figure 5. HDAC4 and HDAC6 associate with HIF-1α protein. *A,* whole-cell protein lysates from C2 cells were immunoprecipitated with rabbit polyclonal antibodies against either HDAC4 or HDAC6 and rabbit IgG as controls. Immunocomplexes were either probed for endogenous HDAC4 or HDAC6 (*left*) or probed for endogenous HIF-1α (*right*). A fraction of the whole-cell lysates without immunoprecipitation was used as input control. *B,* shRNA against HDAC4 and HDAC6. shRNA against HDAC4 and HDAC6 was carried out in ≤50% confluent C2 cells with transfection agent only and shRNA empty vector as negative controls. For each condition, two consecutive transfections were done within 72 hours. Western blot analysis for HIF-1α HDAC4, HDAC6, and Hsp90 was done. *C,* inhibition of HIF-1α transcriptional activity following HDAC4 and HDAC6 knockdown. A similar experiment as described in (*B*) was done. After 72 hours, plasmid p2.1 (2 μg) and pTK-Renilla (0.2 μg) were cotransfected. Dual luciferase activity assays were done 24 hours later. *, $P < 0.05$, versus empty vector control (Student's *t* test). *D,* shRNA induced HIF-1α acetylation. Similar experiments were done as in (*B*) with the final 10 hours in the presence of MG132. Then proteins were immunoprecipitated with anti HIF-1α antibody and blotted with anti–acetyl lysine antibody.

inhibition of HDAC4 and HDAC6 may increase HIF-1α acetylation, shRNA experiments were carried out as shown in Fig. 5*B*. In addition, the proteosome inhibitor MG132 was added in the medium during the final 10 hours of shRNA treatment. Immunoprecipitation of HIF-1α protein revealed similar HIF-1α levels (Fig. 5*D*), suggesting that the proteasome inhibitor rescued HIF-1α from degradation induced by selective HDAC inhibition. There was no significant change in terms of HIF-1α association between Hsp90 and Hsp70 (data not shown). More importantly, Western blot analysis with anti–acetyl lysine specific antibody revealed HIF-1α acetylation in the samples treated with shRNA against HDAC4 (Fig. 5*D*). To our knowledge, this is the first time that HIF-1α is shown to be acetylated after a specific HDAC isozyme inhibition. Western blot of whole-cell lysates also indicated the increase of tubulin acetylation in samples from HDAC6 shRNA. The potential role of microtubule dynamics in HIF-1α stability has previously been reported (22–25).

Discussion

HIF-1α plays a critical role in tumor progression and targeting this transcriptional factor represents an intense field of research

in cancer therapeutics (23, 26, 27). We have previously observed that the HDAC inhibitor LAQ824 targets HIF-1α protein and its downstream VEGF expression in prostate and breast cancer cells (15). In this study, first we investigated whether HIF-1α degradation induced by LAQ824 was due to a VHL-dependent mechanism. Then, by comparison of structurally and functionally different HDAC inhibitors, we hypothesized the involvement of class II HDACs in the protection of HIF-1α stability. For the first time, we observed that HDAC activity was associated with the HIF-1α immunocomplex, and HDAC4 and HDAC6 were associated with HIF-1α.

The lack of selective chemical inhibitors against specific HDAC isozymes initially prevented us from studying the interactions between HIF-1α and specific HDACs using a pharmacologic approach. However, through the use of structurally different HDAC inhibitors and testing the effect on histone acetylation, p21 induction, and HIF-1α inhibition, we observed some clear differences. All inhibitors tested have similar effects on p21 induction and histone hyperacetylation, which are primarily targets of class I HDACs. Interestingly, only hydroxamic acid derivative inhibitors capable of targeting both class I and class II HDACs at low nanomolar concentrations exhibited a significant inhibition of HIF-1α protein expression. Nonhydroxamic acid derivatives, such as valproic acid and benzamide, did not inhibit HIF-1α at concentrations effective against class I HDAC. In contrast, these agents reduced HIF-1α protein expression only at the significantly higher concentrations that are relevant to class II HDACs inhibition. These results lead us to hypothesize the involvement of class II HDACs in HIF-1α regulation. The modest activity of trapoxin in inhibiting HIF-1α suggested the involvement of HDAC6 in HIF-1α stability because this compound has been shown to inhibit class I and class II HDACs, including HDAC4 but not HDAC6 (12).

HDAC7 has been reported to associate with HIF-1α and enhance its nuclear localization and transcriptional activity (28). In C2 cells, we did not observe a clear association between HDAC7 and HIF-1α (data not shown). It is conceivable that the interactions between HIF-1α and HDAC isozymes are different among tumor cell types and under different oxygen tension conditions. There are potential compensatory or redundant mechanisms regulating HDAC isozymes in HIF-1α modulation. HIF-1α sensitivity to hydroxamic acid derivative HDAC inhibitors was consistently observed in normal and transformed cell types. In our immunoprecipitation studies, HIF-1α immunocomplex contained HDAC activity and was found to be associated with both HDAC4 and HDAC6. shRNA against HDAC4 and HDAC6 resulted into both quantitative (protein level) and qualitative (reporter gene expression) inhibition of HIF-1α. Future studies to further elucidate the molecular mechanisms leading to HIF-1α modulation by deacetylase activity of class II HDACs, such as HDAC4 and HDAC6, will be necessary. By using both chemical and biological approaches, our current study suggests a molecular link between HIF-1α and class II HDACs and provides a rationale for using HDAC inhibitors as a novel strategy for targeting HIF-1α.

HIF-1α has been found to be associated with many proteins that modify its stability and function. In our study, both HDAC4 and HDAC6 seem to be new partners linked with HIF-1α stability. Our data suggest that endogenous HIF-1α protein can be acetylated after treatment with LAQ824 and HDAC4-specific shRNA. One possible scenario is that class II HDACs, such as HDAC4, regulate the acetylation level of HIF-1α protein. Hydroxamic acid derivative compounds inhibit the HDAC activity and allow acetyltransferases to increase HIF-1α acetylation. The acetylated HIF-1α then binds to either VHL or some unknown E3 ubiquitin ligase complex for proteasomal degradation. Previously, the Hsp90 inhibitor–initiated HIF-1α degradation in C2 cells was reported as VHL independent (18). The identification of the still elusive E3 ubiquitin ligase complex that compensates the VHL deficiency will be important.

The role of lysine acetylation in protein function and stability has been shown for other proteins such as p53, signal transducers and activators of transcription 3, and NF-κB (7). Lysine acetylation in general promotes protein stability by preventing ubiquitination. The positive association between HIF-1α acetylation and subsequent ubiquitination seems to follow an opposite paradigm. Our results suggest that hydroxamic acid derivative HDAC inhibitors and specific knockdown of HDAC4, but not of HDAC6, induce HIF-1α protein acetylation. The unraveling of the mechanisms responsible for HIF-1α acetylation and eventual ubiquitination will be critical for rational therapeutic strategies targeting this important transcriptional factor.

There may also be some additional mechanisms underlying HIF-1α inhibition induced by class II HDAC inhibitors. HDAC6 has been linked to the chaperon functions of Hsp90 (29, 30). Inhibition of HDAC6 may compromise the HIF-1α stability by also interfering with the chaperon activity of Hsp90 on HIF-1α. Under our experimental conditions, we did not observe a clear shift in HIF-1α association between Hsp90 and Hsp70 at early time points of LAQ824 treatment. HIF-1α protein expression was clearly inhibited following HDAC4 shRNA and this HDAC isozyme has not been directly linked with Hsp90 acetylation. However, our data cannot rule out the possibility that Hsp90 acetylation may still contribute, in part, to HIF-α stability. Another potential pathway mediating the class II HDAC inhibition and HIF-1α degradation is through microtubule dynamics. HDAC6 regulates tubulin dynamics via deacetylation (23, 24). The inhibition of HDAC6 with small interfering RNA and hyroxamic HDAC inhibitors has been shown to alter the microtubule assembly process (23–25). Recently, the alteration of such process has been involved in the HIF-1α inhibition mediated by microtubule-targeting agents (22). Our results suggest that HIF-1α protein expression is inhibited following shRNA against HDAC6 and confirm the role of HDAC6 in tubulin acetylation.

Taken together, our data suggest that class II HDACs associate with HIF-α and are important modifiers of HIF-1α protein stability via a VHL-independent, but proteosome-dependent, pathway. The interaction between HDAC activity and HIF-1α protein stability may be modulated by different HDAC isozymes and via multiple nonexclusive pathways. The definitive mechanism underlying HIF-α modulation by class II HDAC inhibitors remains to be identified but it may include direct HIF-α acetylation, inhibition of transcriptional potential, and disruption of tubulin dynamics and Hsp90 chaperone activity. Additional modifications of HIF-1α induced by class II HDAC may involve protein sumoylation as recently reported for the MEF2 transcription factors (31).

The inhibition of HIF-1α by the hydroxamic acid derivative HDAC inhibitors has clinical implications. Different hydroxamic acid derivative HDAC inhibitors are currently in clinical trials both in solid and hematologic malignancies. At least *in vitro*, plasma achievable concentrations of these agents can induce histone acetylation, growth inhibition, and HIF-1α inhibition. It will be interesting to determine whether HIF-1α and downstream gene

inhibition can also be observed in patients receiving these agents. Inhibition of HIF-1α *in vivo* can impair tumor angiogenesis and circumvent hypoxia-related resistance to chemotherapeutics and radiation (32, 33). Based on these preclinical results, rational combination strategies with hydroxamic acid derivative HDAC inhibitors and molecular targeted therapies warrant clinical testing.

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References

1. Semenza GL. Targeting HIF-1 for cancer therapy. Nat Rev Cancer 2003;3:721–32.
2. Linehan WM, Vasselli J, Srinivasan R, et al. Genetic basis of cancer of the kidney: disease-specific approaches to therapy. Clin Cancer Res 2004;10:6282–9S.
3. Krieg M, Haas R, Brauch H, Acker T, Flamme I, Plate KH. Up-regulation of hypoxia-inducible factors HIF-1α and HIF-2α under normoxic conditions in renal carcinoma cells by von Hippel-Lindau tumor suppressor gene loss of function. Oncogene 2000;19:5435–43.
4. Neckers L, Ivy SP. Heat shock protein 90. Curr Opin Oncol 2003;15:419–24.
5. Jeong JW, Bae MK, Ahn MY, et al. Regulation and destabilization of HIF-1α by ARD1-mediated acetylation. Cell 2002;111:709–20.
6. Eberharter A, Becker PB. Histone acetylation: a switch between repressive and permissive chromatin. Second in review series on chromatin dynamics. EMBO Rep 2002;3:224–9.
7. Sengupta N, Seto E. Regulation of histone deacetylase activities. J Cell Biochem 2004;93:57–67.
8. Grozinger CM, Schreiber SL. Deacetylase enzymes: biological functions and the use of small-molecule inhibitors. Chem Biol 2002;9:3–16.
9. Yang XJ, Gregoire S. Class II histone deacetylases: from sequence to function, regulation, and clinical implication. Mol Cell Biol 2005;25:2873–84.
10. Johnstone RW. Histone-deacetylase inhibitors: novel drugs for the treatment of cancer. Nat Rev Drug Discov 2002;1:287–99.
11. Glaser KB, Li J, Pease LJ, et al. Differential protein acetylation induced by novel histone deacetylase inhibitors. Biochem Biophys Res Commun 2004;325:683–90.
12. Furumai R, Komatsu Y, Nishino N, Khochbin S, Yoshida M, Horinouchi S. Potent histone deacetylase inhibitors built from trichostatin A and cyclic tetrapeptide antibiotics including trapoxin. Proc Natl Acad Sci U S A 2001;98:87–92.
13. Gottlicher M, Minucci S, Zhu P, et al. Valproic acid defines a novel class of HDAC inhibitors inducing differentiation of transformed cells. EMBO J 2001;20:6969–78.
14. Gurvich N, Tsygankova OM, Meinkoth JL, Klein PS. Histone deacetylase is a target of valproic acid-mediated cellular differentiation. Cancer Res 2004;64:1079–86.
15. Qian DZ, Wang X, Kachhap SK, et al. The histone deacetylase inhibitor NVP-LAQ824 inhibits angiogenesis and has a greater antitumor effect in combination with the vascular endothelial growth factor receptor tyrosine kinase inhibitor PTK787/ZK222584. Cancer Res 2004;64:6626–34.
16. Mie Lee Y, Kim SH, Kim HS, et al. Inhibition of hypoxia-induced angiogenesis by FK228, a specific histone deacetylase inhibitor, via suppression of HIF-1α activity. Biochem Biophys Res Commun 2003;300:241–6.
17. Arnesen T, Kong X, Evjenth R, et al. Interaction between HIF-1α (ODD) and hARD1 does not induce acetylation and destabilization of HIF-1α. FEBS Lett 2005;579:6428–32.
18. Isaacs JS, Jung YJ, Mimnaugh EG, Martinez A, Cuttitta F, Neckers LM. Hsp90 regulates a von Hippel Lindau-independent hypoxia-inducible factor-1α-degradative pathway. J Biol Chem 2002;277:29936–44.
19. Semenza GL, Jiang BH, Leung SW, et al. Hypoxia response elements in the aldolase A, enolase 1, and lactate dehydrogenase A gene promoters contain essential binding sites for hypoxia-inducible factor 1. J Biol Chem 1996;271:32529–37.
20. Laughner E, Taghavi P, Chiles K, Mahon PC, Semenza GL. HER2 (neu) signaling increases the rate of hypoxia-inducible factor 1α (HIF-1α) synthesis: novel mechanism for HIF-1-mediated vascular endothelial growth factor expression. Mol Cell Biol 2001;21:3995–4004.
21. Mahon PC, Hirota K, Semenza GL. FIH-1: a novel protein that interacts with HIF-1α and VHL to mediate repression of HIF-1 transcriptional activity. Genes Dev 2001;15:2675–86.
22. Escuin D, Kline ER, Giannakakou P. Both microtubule-stabilizing and microtubule-destabilizing drugs inhibit hypoxia-inducible factor-1α accumulation and activity by disrupting microtubule function. Cancer Res 2005;65:9021–8.
23. Palazzo A, Ackerman B, Gundersen GG. Cell biology: tubulin acetylation and cell motility. Nature 2003;421:230.
24. Matsuyama A, Shimazu T, Sumida Y, et al. *In vivo* destabilization of dynamic microtubules by HDAC6-mediated deacetylation. EMBO J 2002;21:6820–31.
25. Hubbert C, Guardiola A, Shao R, et al. HDAC6 is a microtubule-associated deacetylase. Nature 2002;417:455–8.
26. Rapisarda A, Uranchimeg B, Scudiero DA, et al. Identification of small molecule inhibitors of hypoxia-inducible factor 1 transcriptional activation pathway. Cancer Res 2002;62:4316–24.
27. Pili R, Donehower RC. Is HIF-1α a valid therapeutic target? J Natl Cancer Inst 2003;95:498–9.
28. Kato H, Tamamizu-Kato S, Shibasaki F. Histone deacetylase 7 associates with hypoxia-inducible factor 1α and increases transcriptional activity. J Biol Chem 2004;279:41966–74.
29. Bali P, Pranpat M, Bradner J, et al. Inhibition of histone deacetylase 6 acetylates and disrupts the chaperone function of heat shock protein 90: a novel basis for antileukemia activity of histone deacetylase inhibitors. J Biol Chem 2005;280:26729–34.
30. Kovacs JJ, Murphy PJ, Gaillard S, et al. HDAC6 regulates Hsp90 acetylation and chaperone-dependent activation of glucocorticoid receptor. Mol Cell 2005;18:601–7.
31. Gregoire S, Yang XJ. Association with class IIa histone deacetylases up-regulates the sumoylation of MEF2 transcription factors. Mol Cell Biol 2005;25:2273–87.
32. Semenza GL. Intratumoral hypoxia, radiation resistance, and HIF-1. Cancer Cell 2004;5:405–6.
33. Blagosklonny MV. How avastin potentiates chemotherapeutic drugs: action and reaction in antiangiogenic therapy. Cancer Biol Ther 2005;4:1307–10.