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LW6, a novel HIF-1 inhibitor, promotes proteasomal degradation of HIF-1α via upregulation of VHL in a colon cancer cell line

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ABSTRACT

Hypoxia-inducible factor HIF-1 is responsible for radiation resistance and poor prognosis in cancer therapy. As part of our drug discovery program, a novel HIF inhibitor, LW6, was identified as a small compound that inhibits the accumulation of HIF-1α. We found that LW6 decreased HIF-1α protein expression without affecting HIF-1β expression. MG132, a proteasome inhibitor, protected HIF-1α from LW6-induced proteasomal degradation, indicating that LW6 affects the stability of the HIF-1α protein. We found that LW6 promoted the degradation of wild type HIF-1α, but not of a DM-HIF-1α with modifications of P402A and P564A, at hydroxylation sites in the oxygen-dependent degradation domain (ODDD). LW6 did not affect the activity of prolyl hydroxylase (PHD), but induced the expression of von Hippel-Lindau (VHL), which interacts with prolyl-hydroxylated HIF-1α for proteasomal degradation. In the presence of LW6, knockdown of VHL did not abolish HIF-1α protein accumulation, indicating that LW6 degraded HIF-1α via regulation of VHL expression. In mice carrying xenografts of human colon cancer HCT116 cells, LW6 demonstrated strong anti-tumor efficacy *in vivo* and caused a decrease in HIF-1α expression in frozen-tissue immunohistochemical staining. These data suggest that LW6 may be valuable in the development of a HIF-1α inhibitor for cancer treatment.

1. Introduction

Hypoxia-inducible factor-1 (HIF-1) accumulates in many human tumors and is associated with increased vascular density and tumor grade severity, treatment failure, and a poor prognostic outcome with conventional therapies [1,2]. In animal models, HIF-1 overexpression is associated with increased tumor growth, vascularization, and metastasis, whereas HIF-1 loss-of-function has the opposite effect [3]. HIF-1α has thus become an attractive therapeutic target [1,4]. Under hypoxic conditions, HIF-1α translocates to the nucleus, where it interacts with HIF-1β, p300, and other transcription factors, and binds to hypoxic response element (HRE)-driven promoters [5,6]. HIF-1α up-regulates a number of target genes involved in angiogenesis, survival, metastasis, and cell cycle regulation, to promote survival in low-oxygen conditions [7,8].

HIF inhibitors have been extensively studied [9–12]. A number of novel anticancer agents inhibit HIF-1α through a variety of molecular mechanisms, including transcriptional regulation, protein folding, stabilization, nuclear translocation, degradation, and trans-activation [3,13]. Several mechanisms have been suggested to explain the degradation pathway of the HIF-1α protein. The first mechanism induces oxygen (O_2)-dependent degradation of the HIF-1α subunit during normoxia, mediated by both prolyl hydroxylase (PHD) and the von Hippel-Lindau tumor suppressor (VHL)/Elongin-C/Elongin-B (VBC) E3 ubiquitin ligase

Abbreviations: VHL, von-Hippel-Lindau; VBC, von Hippel-Lindau (VHL)/Elongin-C/Elongin-B; ODDD, oxygen-dependent degradation domain; 17-AAG, 17-allylamino-geldanamycin; VEGF, vascular endothelial growth factor; DIP, dipyridol; PHD, prolyl hydroxylase; HUVEC, human umbilical vascular endothelial cells; HSP90, heat-shock protein 90.

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complex. HIF-1α is hydroxylated at proline residues in the oxygen-dependent degradation domain (ODDD) by three prolyl hydroxylases, PHD1–3. These modifications allow HIF-1α to bind to VHL [14–16], which associates with VCB-Cul2 E3 ligase for proteasomal degradation [17,18]. The second proposed mechanism involves p53-mediated accelerated degradation of HIF-1α [19]. Finally, the inhibition of heat-shock protein 90 (HSP90) leads to O_2/PHD/VHL-independent degradation of HIF-1α, and the receptor of activated protein kinase C (RACK1) is shown to promote PHD/VHL-independent proteasomal degradation of HIF-1α. The HSP90 inhibitor, 17-allylaminogeldanamycin (17-AAG), degrades HIF-1α by binding directly to RACK, which binds to elongin C and promotes HIF-1α ubiquitination [20].

Previously, we reported the synthesis of a novel compound, an (aryloxyacetylamino)benzoic acid derivative, LW6, which inhibits the accumulation of HIF-1α [21]. Here, we present evidence that LW6 promotes HIF-1α degradation via upregulation of VHL. LW6 exhibited 53.6% tumor growth inhibition in a *in vivo* xenograft assay using the HCT116 cell line. These results suggest that LW6 may serve as a candidate in the development of a HIF-1α inhibitor for cancer therapy.

2. Materials and methods

2.1. Materials

An (aryloxyacetylamino)benzoic acid derivative LW6 (Fig. 1A) was synthesized as described (compound 23 [21]). Chemicals were purchased from Life Technologies Inc. (Gaithersburg, MD, USA), and media was obtained from Sigma (St. Louis, MO, USA). Cell culture media and fetal bovine serum (FBS) were purchased from Gibco Laboratories (Grand Island, NY, USA). A Phototope-Horseradish Peroxidase Western Blot Detection Kit was obtained from Millipore (Billerica, MA, USA). The growth medium and the supplements for HUVEC were purchased from Lonza Walkersville Inc. (Walkersville, MD, USA). Matrigel and reagent for the *in vitro* tube formation assay were purchased from BD Biosciences (San Diego, CA, USA). siRNAs were synthesized for knockdown assays (Samchully Pharm. Inc. Co., Korea). Rabbit polyclonal antibody against GAPDH was from AB Frontier (Seoul, Korea), and β-actin was supplied by ABM Inc. (Brampton, Canada). Mouse monoclonal antibodies (MAb) against VHL, anti-HIF1β and anti-HIF-1α were obtained from BD Biosciences (San Diego, CA, USA), and anti-HA tag antibody was purchased from Cell Signaling (Danvers, MA, USA).

2.2. Cell culture

HCT116 (human colorectal carcinoma), Caki-1 (human Caucasian kidney carcinoma), SK-HEP1 (hepatocellular carcinoma), and PC-3 (human prostate carcinoma) cells were cultured in RPMI 1640 with 10% fetal bovine serum (FBS; Lonza Walkersville Inc., Walkersville, MD, USA). Human umbilical vein endothelial cells (HUVEC) were grown in EBM-2 medium supplemented with the bullet kit growth supplement mixture (0.5 ml hEGF, 0.2 ml hydrocortisone, 0.5 ml GA-1000, 2 ml hFGF-B, 0.5 ml VEGF, 0.5 ml R3-IGF-1, 0.5 ml ascorbic acid, 0.5 ml heparin and 10 ml FBS in 500 ml). All media contained 100 U/ml penicillin and 100 μg/ml streptomycin. Cancer cell lines were obtained from the ATCC and HUVEC was purchased from Lonza Walkersville Inc. (Walkersville, MD, USA). All cells were cultured in an atmosphere of 5% CO_2 at 37 °C, and hypoxia was induced by culturing cells in a hypoxia chamber (Sanyo O_2/CO_2 incubator, MCO-18M, Sanyo Electric Co. Ltd., Japan) flushed with a mixed gas of 1% O_2, 5% CO_2, and 94% N_2.

2.3. Western blot analysis

Cells were lysed with RIPA buffer (Millipore) containing protein inhibitors (Roche Diagnostics, Mannheim, Germany), and 1 mM each of NaF, Na_3VO_4, DTT, and PMSF. Cell extracts were prepared and Western blots analysis was carried out as described [22].

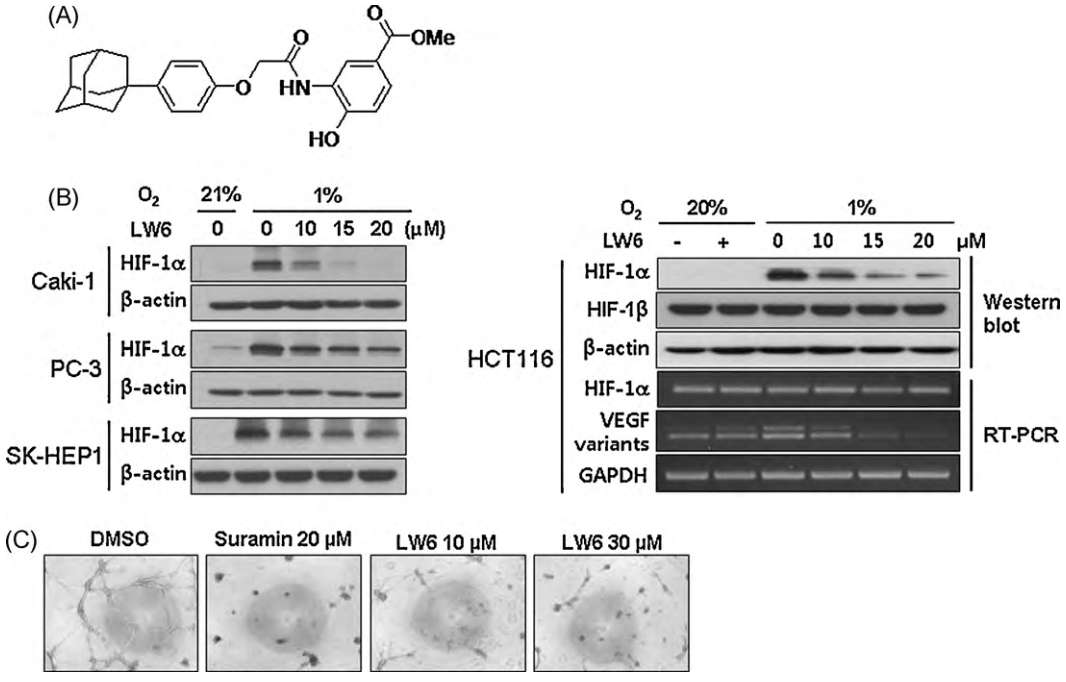

Fig. 1. LW-6, a HIF-1α inhibitor. (A) Structure of LW6, 3-[2-(4-adamantan-1-yl-phenoxy)-acetylamino]-4-hydroxy-benzoic acid methyl ester (compound 23) [21]. (B) The effect of LW6 on HIF-1α protein expression and its target gene. Cancer cells, Caki-1, PC-3, SK-HEP1, and HCT116, pre-incubated under hypoxia for 4 h were treated with LW6 (0, 10, 15, and 20 μM) for 12 h. The effect of LW6 on HIF-1α protein expression was determined by Western blot analysis, and VEGF transcript level was quantified by RT-PCR. (C) *In vitro* tube formation assay. Human umbilical vein endothelial cells (HUVECs) were seeded in plates coated with ice-cold BD Matrigel™ matrix gel solution, and tubule branches were observed in the presence and absence of LW6 (10 and 30 μM). Suramin (20 μM) was used as a positive control.

2.4. RNA extraction and RT-PCR

Total RNA was purified from HCT116 cells using a Qiagen RNeasy Mini kit (Qiagen, Hilden, Germany) and mRNA levels were measured by RT-PCR [23]. The primer sequences were as follows: vascular endothelial growth factor (VEGF), forward, 5′-GGTGGACATCTTCCAGAGTA-3′, reverse, 5′-GGCTTGTCACATCTGCAAGTA-3′; erythropoietin (EPO), forward 5'TATGCCTGGAAGATGGAGGTC-3′, reverse 5′-TGTCAGCAGTGATTGTTCGGAAG-3′; HIF-1α, forward 5′-CTATATCCCAATGGATGATGATGA-3′, reverse 5′-ATCATGTTCCATTTTTCGCTT-3′; glyceraldehyde 3-phosphate dehydrogenase (GAPDH), forward 5′-ATGGGGAAGGTGAAGGTCGG-3′, reverse 5′-CAGGAGGCATTGCTGATGAT-3′.

2.5. In vitro tube formation assay

Human umbilical vein endothelial cells (HUVEC), growth medium and supplements were purchased from Lonza Walkersville Inc. (Walkersville, MD, USA). For *in vitro* tube formation assays, wells of a 96-well plate were coated with ice-cold BD Matrigel™ matrix gel solution. After polymerizing the matrix at 37 °C, HUVECs (passages 3–5) were seeded onto the polymerized EC matrix at a concentration of 1×10^4 cells in 180 μl of EBM-2 medium supplemented with a growth supplement mixture (0.5 ml hEGF, 0.5 ml hydrocortisone, 0.5 ml GA-1000, 2 ml BBE and 10 ml FBS in 500 ml) per well. Test compound in 20 μl of EMB-2 medium was immediately added to each well. The tubule branches were photographed after 16 h of incubation as described [24,25].

2.6. Reporter assay

Inhibition of HIF-1α was assayed by a reporter assay using dual-luciferase reporter assay system (Promega, Madison, WI, USA), as previously described [26]. HCT116 cells in 75–90% confluence were transiently co-transfected with pGL3-HRE-luciferase plasmid containing six copies of HREs from human *VEGF* genes and pRL-SV40 encoding firefly renilla luciferase and incubated for 24 h. Cells were treated with LW6 or 17-AAG for 16 h before report assay. Luciferase activity was integrated over a 10 second period and measured using a luminometer (Victor X Light; PerkinElmer, Waltham, MA, USA). The results were normalized to the activity of renilla luciferase. Data are presented as means ± SD.

2.7. Plasmid construction

To construct a plasmid expressing the doubly mutated HIF-1α with alanine substitutions at the Pro402 and Pro564 residues, a cDNA was amplified using forward primer CTTTGCTGGCCGCAGCCGCTGGAG (the underlined base: P402A mutation) and reverse primer GGGATATAGGCAGCTAACATCCTT (the underlined base: P564A mutation) and template pcHIF-1α containing the full length HIF-1α cloned into pcDNA3.1+. The resulting cDNA was purified and used as a primer with pcHIF-1α as a template to construct the doubly mutated pcHIF-1α (pcHIF-DM) using the Stratagene QuickChange site-directed mutagenesis kit (Stratagene, USA). The mutated bases were confirmed by DNA sequencing. To generate HA tagged HIF-1α, dm-HIF1α and wt-HIF-1α were subcloned into pCMV-beta-HA. HA-tagged K532R HIF-1α (K532R HIF-1α) was constructed by the same method, using a forward primer 5′-GTGATATGGTCAATGAATTCAGGTTGGAATTGGTAGAAAAAC-3′ (the underlined base: K532R mutation).

2.8. In vitro binding assay

The peptide substrates and GST-VBC proteins required for the binding assay were prepared as previously described [27]. Fluorescence-labeled peptide substrate F-P564 (FITC-ACADLDLEALAPYIPADDDFQLR; final concentration 1 μM) was incubated with 0.2 μg/μl recombinant His_6-PHD2 in NETN buffer containing 2 mM ascorbic acid, 5 mM 2-oxoglutarate, 100 μM $FeCl_2$, and 5 μM N,N,N′,N′-tetrakis (2-pyridylmethyl) ethylenediamine (TPEN), with increasing concentrations of LW6 for 30 min at 25 °C. A reaction mixture containing DMSO alone without His_6-PHD2 was included as a negative control, and another control contained F-HyP564 (a fluorescently labeled peptide with hydroxylation at P564). The reactions were terminated by heating for 1 min at 95 °C, followed by dilution to a final peptide concentration of 100 nM in EBC buffer (50 mM Tris, pH 8.0, 120 mM NaCl, 0.25% Nonidet P40) in the presence of 500 nM GST-VBC. Fluorescence polarization values were measured using a Luminescence Spectrometer LS50B (PerkinElmer).

2.9. siRNA knockdown assay

An siRNA knockdown assay was carried out as previously described [22]. The siVHL sequence was 5′-CGC AUU GCA CAU CAA CGG ATT-3′. As a control, siGFP 5′-GUU CAG CGU GUC CGG CGA CTT-3′ was used. siRNAs were transfected into HCT116 cells (20 nM) using lipofectamine LTX reagent (Invitrogen, Carlsbad, CA) and incubated for 24 h. Then, cells pre-incubated under hypoxia for 4 h were treated with LW6 or 17-AAG for 12 h to analyze the effect of knockdown of VHL by Western blot.

2.10. In vivo xenograft assay

Six weeks old specific-pathogen-free Crj:BALB/c nu/nu female athymic nude mice (Charles River, Japan) were kept in an autoclaved cage to avoid contact with pathogens. All animal experiments were approved by the Institutional Animal Care and Use Committee, the Korea Research Institute of Bioscience and Biotechnology (Daejeon, Korea). All mice were adapted to the sterile basic supplementary diet and acclimated to laboratory conditions for a week before use. To establishe human colorectal carcinoma tumors in mice, HCT116 cells were cultured in RPMI-1640 medium containing 10% heat-inactivated fetal bovine serum under a humidified atmosphere of 5% CO_2 at 37 °C. The cells were then detached by trypsinization, washed, and resuspended in phosphate-buffered saline. The mice were randomly assigned to three groups, each of which consists of six mice ($n = 6$ per group), and then were subcutaneously inoculated with 0.3 ml of HCT116 cells (4×10^7 cells/ml) in the right flank region. When the tumor volume reached approximately 100 mm^3, the mice received the following treatments using a dosing vehicle solution, containing 10% dimethylacetamide, 10% Cremophor EL and 80% of sodium carbonate buffer (pH 10), by intraperitoneal (i.p.) injection: group 1 (control group; six mice), vehicle solution; group 2 (six mice), LW6 at a dose of 10 and 20 mg/kg (QD); and group 3 (six mice), topotecan at a dose of 2 mg/kg, (Q2D), which is the dose and dosing schedule that showed more than 60% inhibition of growth of HCT116 tumors. The treatments were continued for 13 days. Tumor volume (V) was determined using the following equation: $V = (L \times W^2) \times 0.5$, where L is the length of the long side of the tumor and W is the length of the short side. Tumor growth inhibition was analyzed for statistical significance using ANOVA followed by Dunnett's pairwise t-test. A p-value of <0.05 was considered statistically significant. Data are presented as means ± SD.

3. Results

3.1. LW6 inhibits HIF-1α protein accumulation and suppresses the expression of hypoxia-induced genes

HIF-1α protein expression increases under hypoxic conditions, while remaining at a baseline detectable level in

normoxia. LW6, an (aryloxyacetylamino)benzoic acid derivative (Fig. 1A), inhibited accumulation of HIF-1α protein in human cancer cell lines, Caki-1, PC-3, SK-HEP1 and HCT-116 (Fig. 1B). Human colorectal carcinoma cells HCT116 was chosen for further study because HCT116 showed dramatic accumulation of HIF-1α protein during hypoxia and distinct reduction of HIF-1α protein in the presence of LW6. Moreover, *in vivo* xenograft analysis with HCT116 can be easily performed compared to that with Caki-1 cells.

The accumulation of HIF-1α protein is almost abolished by treatment with LW6 in both a dose- and time-dependent manner in HCT116 (Fig. 1B, Supplementary Fig. 1). HIF-1α mRNA levels remained unchanged by LW6, indicating that LW6 is a post-transcriptional regulator. In contrast, the level of HIF-1β protein remains constant in the presence of LW6 (Fig. 1B). LW6, therefore, specifically inhibits the accumulation of HIF-1α in the HCT116 under hypoxic conditions.

Transcript level of VEGF decreased with increasing HIF-1α protein level, in the presence of LW6 (Fig. 1B). The effect of LW6 on VEGF was also assessed by *in vitro* tube formation, a simple model for angiogenesis as a biological function of VEGF. When human umbilical vascular endothelial cells (HUVEC) were grown on a Matrigel surface, the capillary-like structures of normal pro-angiogenesis were observed. However, *in vitro* tube formation was inhibited when HUVEC were incubated on Matrigel in the presence of 10 μM LW6 for 16 h (Fig. 1C). There were no cytotoxic effects associated with LW6 at this concentration (Supplementary Fig. 2). LW6 does not seem to be a cytotoxic drug because it induced gradual growth inhibition of HUVEC and HCT116 cells high concentration ($GI_{50} > 40$ μM, while Topotecan caused dramatic cell death ($GI_{50} < 0.05$ μM, Supplementary Fig. 3). These results demonstrate that LW6 significantly inhibits hypoxia-induced accumulation of HIF-1α, leading to suppression of transcription and function of VEGF in tube formation of HUVEC.

3.2. LW6 influences the degradation pathway of the HIF-1α protein

Having established that LW6 regulation of HIF-1α is post-transcriptional, we next examined the effect of LW6 on the degradation of the HIF-1α protein. Treatment with MG132, a proteasomal inhibitor (Z-Leu-Leu-Leu-Al), prevents degradation of ubiquitinated HIF-1α protein in both normoxia and hypoxia (Fig. 2A). In the absence of MG132, HIF-1α accumulation during hypoxia was abolished by LW6. In contrast, HIF-1α protein accumulation was unaffected by LW6 in the presence of MG132, suggesting that LW6 promotes proteasomal degradation of HIF-1α. MG132 prevention of HIF-1α proteasomal degradation resulted in considerable accumulation of HIF-1α, to a greater extent than typically observed under hypoxia.

3.3. Inhibition of prolyl hydroxylase suppresses LW6-induced proteasomal degradation of HIF-1α

Mdm2-mediated binding of p53 to HIF-1α causes ubiquitination and degradation of HIF-1α [19,28]. LW6 reduces p53 expression, suggesting that HIF-1α degradation does not involve p53-Mdm2 complex (Supplementary Fig. 4). To investigate whether the degradation of HIF-1α by LW6 requires prolyl hydroxylation of P402 and P564 in the ODDD, HRE-luciferase reporter assays were carried out in the presence of dipyridol (DIP), a prolyl hydroxylase inhibitor (Fig. 2B). Luciferase activity increased in the presence of DIP, which inhibited hydroxylation of P402 and P564 in the ODDD. As expected, LW6 reduced HRE-Luc activity in the absence, or at low concentrations, of DIP. However, LW6 did not reduce HRE activity when prolyl hydroxylation of HIF-1α was inhibited by 200 μM DIP. Destabilization of HIF-1α by LW6, therefore, seems to be associated with prolyl hydroxylation. In other words, LW6 requires HIF-1α to be prolyl hydroxylated for activity. The HRE reporter assay was carried out using 17-AAG, which leads to O_2/PHD/VHL-independent degradation of HIF-1α.

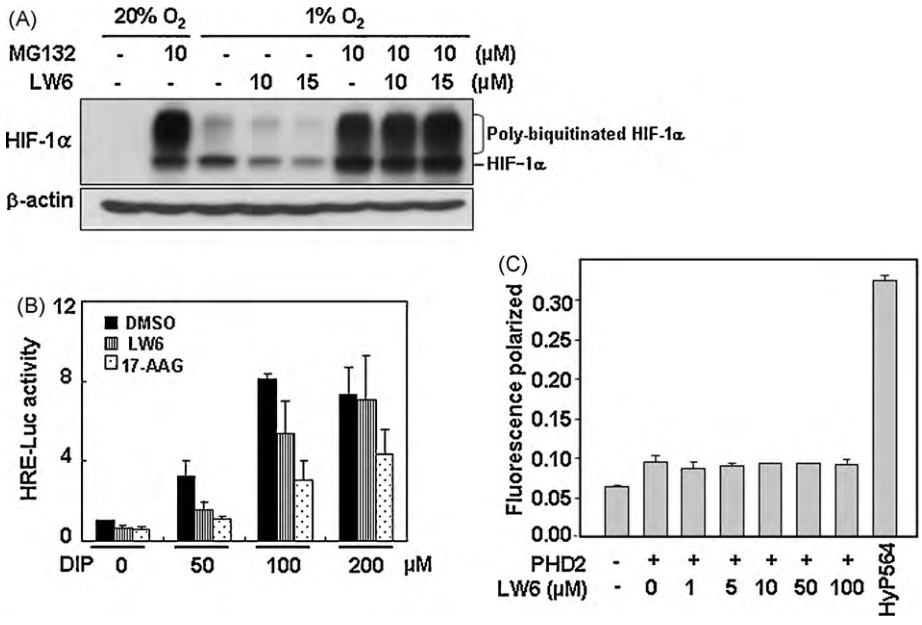

Fig. 2. Effect of LW6 on proteasomal degradation of HIF-1α. (A) Proteasomal degradation of HIF-1α in the presence of LW6. HCT116 cells were treated with LW6 (0, 10, and 15 μM) for 4 h and further incubated for 8 h in the presence of 10 μM MG132. (B) The effect of LW6 on HRE-luciferase activity in the presence of DIP. Cells co-transfected with pGL3-HRE-luciferase and pRL-SV40 were incubated for 24 h and treated with LW6 (15 μM) or 17-AAG (0.4 μM) in the presence of DIP (0, 50, 100, and 200 μM) for 12 h for the luciferase assay. (C) The effect of LW6 on the activity of prolyl hydroxylase 2 (PHD2). The fluorescent HIF-1α peptide (1 μM) was incubated with PHD2 (0.2 μg/μl) in the presence of LW6 (0, 1, 5, 50, and 100 μM) for 30 min and allowed to bind purified VBC protein; this was followed by determination of polarized fluorescence using the LS50B Luminescence Spectrometer (PerkinElmer).

Luciferase activity was reduced in the presence of 17-AAG, regardless of the presence of DIP (Fig. 2B), thus indicating that inhibition of prolyl hydroxylation suppresses LW6-induced proteasomal degradation of HIF-1α.

3.4. LW6 did not affect in vitro activity of prolyl hydroxylase

We next addressed the possible effect of LW6 on prolyl hydroxylase 2 (PHD2) activation by *in vitro* binding assays using a fluorescent HIF-1α peptide containing the proline 564 residue required for binding to VBC (pVHL/elongin B/elongin C) [27]. The HIF-1α peptide was incubated with PHD2 in the presence of LW6 and allowed to bind to purified VBC protein, followed by polarized fluorescence detection as a measure of the binding affinity of HIF-1α to VBC (Fig. 2C). Hydroxylated P564, a positive control, showed high VBC binding activity. Binding of HIF-1α peptide to VBC increased in the presence of PHD2, presumably due to PHD2-dependent hydroxylation of the peptide at Pro564. However, increasing concentrations of LW6 did not increase the binding of HIF-1α peptide to VBC in the presence of PHD2. A similar result was obtained when PHD3 was used instead of PHD2 (Supplementary Fig. 5), indicating that LW6 was not involved in the activation of PHD for prolyl hydroxylation of HIF-1α.

3.5. The hydroxylation at P564 and P402 in the ODDD is essential for LW6-induced degradation of HIF-1α

To confirm that hydroxylation of HIF-1α P564 and P402 is essential for LW6-induced degradation of HIF-1α, we constructed a DM-HIF-1α mutant containing P564A and P402A mutations in the ODDD and tested its function in the HRE reporter assay (Fig. 3). 17-AAG was used as a control. When the HIF-1α gene (WT-HIF-1α) was co-transfected with HRE-Luc vector in the assay, luciferase activities in the presence of LW6 and 17-AAG decreased by 56% and 47% respectively, indicating similar effects of LW6 and 17-AAG on degradation of wild type HIF-1α in those conditions (Fig. 3A). Co-transfectants containing DM-HIF-1α showed a 6.3-fold increase in HRE activity, indicating that prevention of hydroxylation at both P564 and P402 is essential for HIF-1α accumulation. Interestingly, LW6 slightly decreased HRE-luciferase activity, whereas activity was 50% reduced by 17-AAG in the presence of hydroxylation-negative HIF-1α. HIF-1α hydroxylation is, therefore, essential for its degradation by LW6.

The lysine at position 532 in the HIF-1α ODDD is acetylated by ARD1 acetyltransferase [14]. Because this modification is known to facilitate HIF-1α recognition by VHL, the importance of acetylation to LW6 degradation of HIF-1α was examined using a K532R HIF-1α mutant as shown in Fig. 3Ab. Acetylation at K532 had little effect on HIF-1α degradation; HRE-luciferase activity in cells containing K532R HIF-1α increased only 1.2-fold over the activity in cells containing WT-HIF-1α. Both LW6 and 17-AAG decreased HRE-luciferase activity in the presence of either Wt-HIF-1α or K532R-HIF-1α. These result suggest that acetylation at K532 in the ODDD is not important for LW6-dependent proteasomal degradation of HIF-1α.

To further verify that LW6 causes HIF-1α degradation, cells transfected with HA-labeled WT-HIF-1α or DM-HIF-1α, were treated with LW6 or 17-AAG, and HIF-1α expression was monitored with anti-HA antibody (Fig. 3B). DM-HIF-1α was expressed at a greater level, due to mutations of the hydroxylation sites in the ODDD, while WT-HIF-1α protein was not. As expected, 17-AAG had similar, O_2/VHL-independent effects on the degradation of both WT and DM-HIF-1α. However, LW6 treatment led to degradation of WT-HIF-1α, but not DM-HIF-1α, indicating that LW6 specifically promotes degradation of prolyl-hydroxylated HIF-1α.

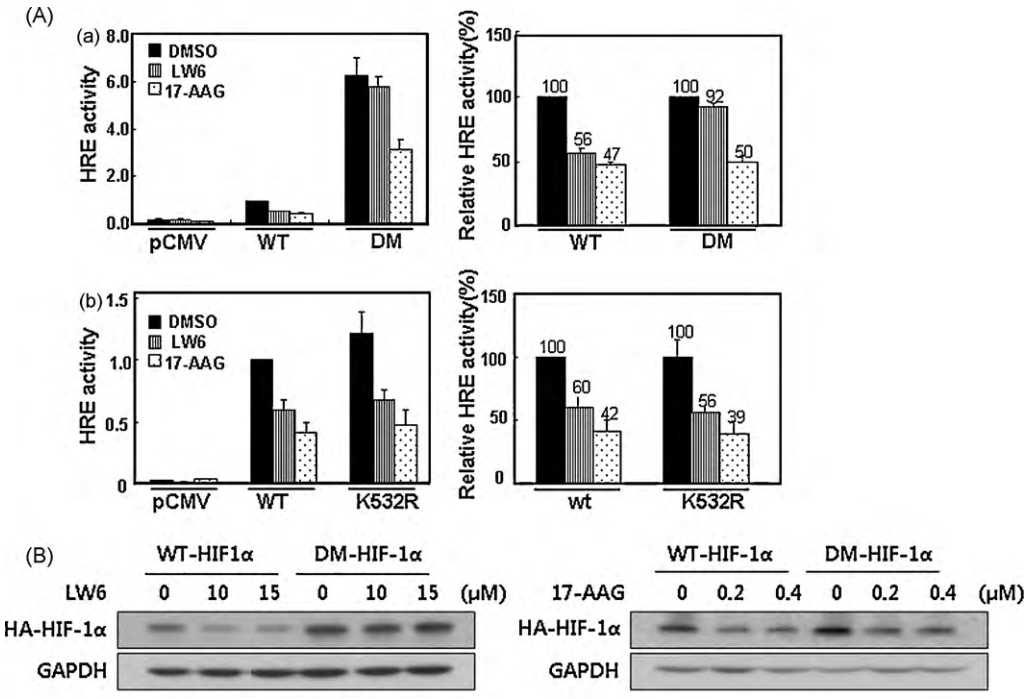

Fig. 3. Effect of LW6 on modification of HIF-1α in the ODDD. (A) Effects of hydroxylation (a) and acetylation (b) of the ODDD on LW6-mediated degradation of HIF-1α. To study the effect of hydroxylation, cells were co-transfected with plasmids, pGL3-HRE-Luc, pRL-SV40, and pCMV-wt-HIF1α, or pCMV-DM-HIF-1α (P402A, P564A), and were incubated for 24 h (a). To study the effect of acetylation, cells were co-transfected with plasmids, pGL3-HRE-Luc, pRL-SV40, and pCMV-wt-HIF-1α, or pCMV-K532R-HIF1α (b). Cells were then treated with LW6 (15 μM) or 17-AAG (0.4 μM) for 12 h. Luciferase activity (left) and the relative value of luciferase activity (right) compared to those of the untreated cells are presented. (B) Western blot analysis of cells treated with LW6 as above (Aa). Extracts were prepared from cells treated for the hydroxylation assay in the presence of LW6 or 17-AAG.

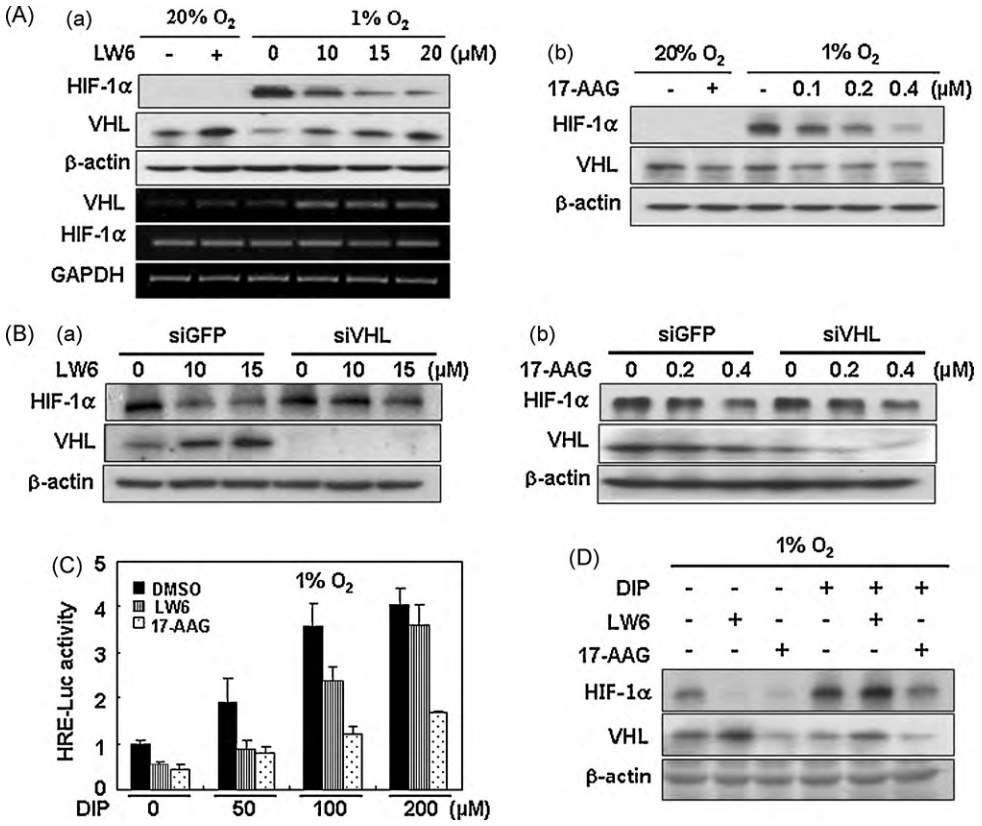

Fig. 4. LW6 increased VHL expression. (A) The effects of LW6 (a) and 17-AAG (b) on VHL expression. HCT116 cells were treated with LW6 (0, 10, 15, and 20 μM) or 17-AAG (0, 0.1, 0.2, and 0.4 μM) for 12 h under hypoxia, and samples were prepared for Western blot and RT-PCR analyses (B) VHL knockdown effect on the degradation of HIF-1α by LW6. An siRNA knockdown assay was carried out using siVHL (20 nM) and siGFP (20 nM). HIF-1α expressions were examined in the VHL-knockdown cells treated with either LW6 (a) or 17-AAG (b) for 16 h. (C) The effects of LW6 on HRE-luciferase activity and VHL expression in the presence of DIP. The cells co-transfected with pGL3-HRE-luciferase and pRL-SV40 were incubated for 24 h. After incubating cells in hypoxia for 4 h, they were treated with 15 μM LW6 or 0.4 μM 17-AAG in the presence of 200 μM DIP for 12 h under hypoxia. Luciferase activity and Renilla activity were measured. (D) Western blot analysis. HCT116 cells treated with 10 μM LW6 or 0.4 μM 17-AAG in the presence of 200 μM DIP under hypoxia were lysed with the RIPA buffer, and total lysates were prepared.

3.6. LW6 up-regulates VHL expression and interaction with prolyl-hydroxylated HIF-1α

Using transient expression of HIF-1α and VHL, we confirmed that increasing VHL expression caused rapid degradation of HIF-1α (Supplementary Fig. 6). We then investigated the effect of LW6 on VHL expression and were surprised to find that LW6 treatment caused a dose- and time-dependent increase in both the VHL protein and transcript levels in normoxia and hypoxia, indicating that LW6 regulates VHL expression at the transcriptional level (Fig. 4A, Supplementary Fig. 7). In contrast, cells treated with 17-AAG showed slightly reduced VHL expression (Fig. 4Ab). It is not clear how 17-AAG decreases VHL expression.

VHL siRNA knockdown and HIF-1α protein expression analysis was used to address whether LW6 inhibits HIF-1α accumulation via VHL expression activation (Fig. 4B). In the presence of LW6, VHL expression increased and HIF-1α expression decreased in cells treated with siGFP. LW6 did not affect HIF-1α expression in VHL-knockdown cells, whereas 17-AAG did reduce HIF-1α expression. We conclude, therefore, that LW6 destabilizes the HIF-1α protein by increasing VHL expression in HCT116 cells.

Next, we compared the effects of LW6 and 17-AAG on HIF-1α and VHL expression during hypoxia using an HRE-luciferase assay (Fig. 4C) and Western blot analysis (Fig. 4D). It was interesting to observe that HRE-Luc activity was induced by increasing concentrations of DIP, even during hypoxia (Fig. 4C), in spite of previous reports of prolyl hydroxylase O_2 dependence. Prolyl hydroxylation of HIF-1α seems to be catalyzed even at very low oxygen levels (1% O_2). As expected, LW6 reduced HRE activity by about 50% in the absence of DIP. However, cells pre-incubated with DIP showed a dose-dependent increase in HRE activity, suggesting that a inhibition of hydroxylase activity induced HIF-1α accumulation. Therefore, HRE-luciferase activity of cells pretreated with 200 μM DIP was substantially increased even in the presence of LW6. Cells treated with 17-AAG, however, had greatly decreased HRE-luciferase activity, at high concentration of DIP. HRE-luciferase activity determined in the presence of LW6 or 17-AAG was consistent with HIF-1α protein expression levels, as demonstrated by Western blot analysis (Fig. 4D). VHL expression was induced only when cells were treated with LW6, regardless of the presence of DIP. Although VHL expression increased in the presence of LW6, HRE activity did not increase in the presence of DIP, due to inhibition of HIF-1α prolyl hydroxylation. This result clearly indicates that LW6 promotes HIF-1α degradation via upregulation of VHL, which interacts with prolyl-hydroxylated HIF-1α for ubiquitination and subsequent proteasomal degradation.

3.7. LW6 inhibits growth of HCT116 cells in a xenograft tumor model

To evaluate the *in vivo* anti-tumor activity of LW6, we generated tumors in athymic nude mice by subcutaneous inoculation of the human cancer cell line HCT116. When the tumor volume reached ~100 mm^2, tumor-bearing mice were treated with LW6 (10 and 20 mg/kg) every day until the end of the study (Fig. 5). The administration of LW6 (20 mg/kg) significantly inhibited HCT116 tumor growth up to 53.6%, compared to that of the vehicle-treated control group (Fig. 5A). LW6-treated daily at a dose of 10 mg/kg

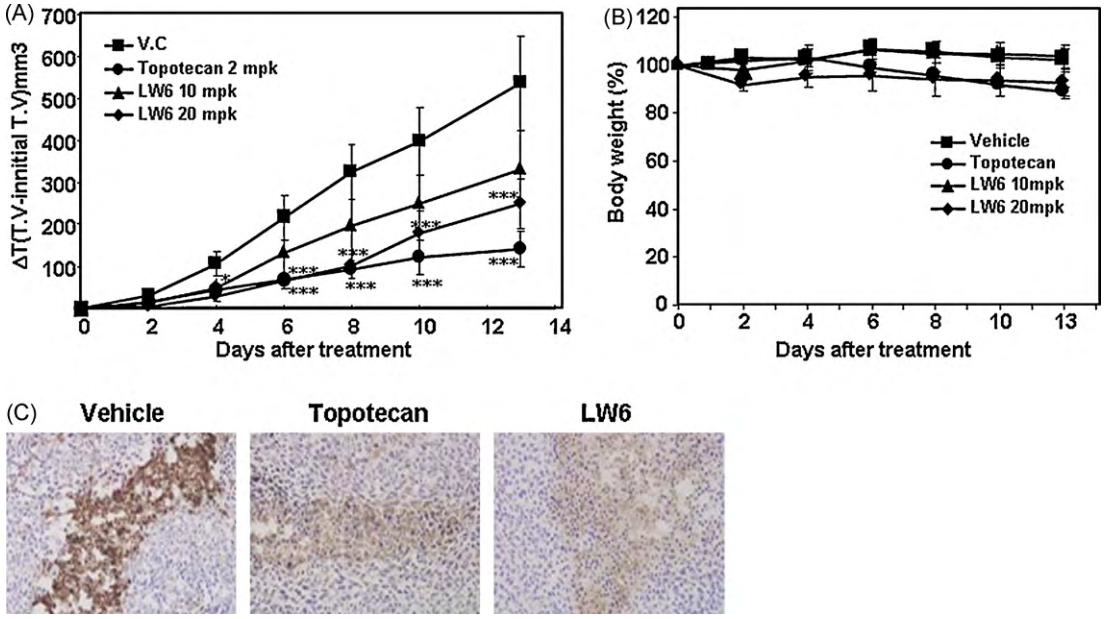

Fig. 5. *In vivo* anti-tumor activity of LW6. HCT116 cells were injected subcutaneously to generate tumors in nude mice. When the tumor volume reached ~100 mm^2, tumor-bearing mice were treated with LW6 (10 and 20 mg/kg, QD) or Topotecan (2 mg/kg, Q2D) for 13 days. Data are means ± SD. Significance was determined using Dunnett's *t*-test vs. the vehicle group ($^{*}p < 0.05$, $^{***}p < 0.001$). (A) Effect of LW6 on tumor growth inhibition. (B) Changes in body weight. (C) Immunohistochemical staining of frozen, untreated, and topotecan- and LW6-treated tissues in the xenograft with the anti-HIF-1α antibody.

produced 37.4% inhibition of tumor growth. These results suggest that LW6 is able to inhibit tumor growth dose-dependently. Topotecan, a clinical compound that suppresses both HIF-1α and HIF-2α protein accumulation [29], inhibited tumor growth by 73.7%. LW6 treatment did not cause significant weight loss or side effects, such as skin ulcers or other severe symptoms (Fig. 5B).

To address whether the anti-tumor effect of LW6 was due to a decrease in HIF-1α, frozen sections of day 14 xenografts were processed for immunohistochemical staining with anti-HIF-1α antibody (Fig. 5C). The sections of tumors treated with either topotecan or LW6 showed low levels of HIF-1α, whereas high levels of HIF-1α were detected in untreated tumor tissue. This result further supports LW6, a HIF inhibitor, as a potential lead compound for cancer therapy.

4. Discussion

Many attempts have been made to identify small molecules that inhibit the accumulation of HIF-1α, a key player in tumorigenesis under hypoxia. Herein, we present LW6, a HIF-1α inhibitor that abolished the accumulation of HIF-1α protein and led to suppression of its target genes under hypoxia. Prolyl hydroxylation at P564 and P402 in the ODDD is a major process for O_2-dependent ubiquitination and degradation of HIF-1α. The increase in HRE-Luc activity, even during hypoxia, with increasing concentrations of DIP, a PHD inhibitor, suggests the presence of PHD activity at very low levels of oxygen (1% O_2). In fact, it has been shown that induction of PHD2 protein expression in response to hypoxia and NO [30,31].

It is clear that LW6 only accelerated the proteasomal degradation of wild type HIF-1α by increasing VHL expression. LW6 could not induce degradation of a HIF-1α mutant that had been modified at the P402A and P564A hydroxylation sites in the oxygen-dependent degradation domain (ODDD). However, acetylation at Lys532 in the ODDD did not affect HIF-1α degradation by LW6 as shown in the assay with K532R-HIF-1α mutation. We finally found that LW6 up-regulates VHL expression, which promotes HIF-1α proteasomal degradation by interacting with prolyl-hydroxylated HIF-1α for ubiquitination.

Germ-line mutations in the VHL gene can be found in patients with VHL disease, a rare familial tumor syndrome characterized by the development of highly vascularized tumors in multiple organs [32]. VHL is expressed in most tissues and cell types [33], and loss of pVHL may be generally involved in tumor progression and angiogenesis [34,35]. Inhibition of E2-EPF ubiquitin carrier protein (UCP) to target VHL for ubiquitin-mediated proteolysis in cells results in destabilization of HIF-1α [36]. The adenovirus expressing VHL lead to a significant reduction in VEGF expression *in vitro* and adVHL effectively inhibits angiogenesis in retina and iris in laser-induced multiple BRVO in monkey eyes [37]. Non-steroidal anti-inflammatory drugs (NSAIDs) increase VHL expression, leading to decreased HIF-1α accumulation and inhibition of hypoxia-induced angiogenesis [38]. Therefore, regulation of VHL expression during hypoxia can provide an effective cancer therapy for solid tumors. LW6 can function as a HIF-1 inhibitor.

A remaining question to address is how LW6 increases VHL expression. There are several possibilities: LW6 may regulate VHL by epigenetic control, such as demethylation of the VHL promoter or inhibition via histone deacetylation. The VHL promoter of VHL is reported to be hypermethylated in many cancer tissues [39,40], and that promoter hypomethylation results in increased gene expression. LW6 may also target a transcription factor or regulatory molecule that activates VHL transcription. Otherwise, LW6 may up-regulate VHL via suppression of its proteasomal degradation by interfering E2-EPF.

Like other HIF-1α inhibitors, such as those that inhibit topoisomerase I [41] and II [42], phosphatidylinositol 3-kinase [43], and heat shock protein 90 [20], LW6 is capable of functioning in a cancer cell line. Furthermore, LW6 exhibited potent *in vivo* efficacy, presumably via suppression of HIF-1α expression, which was further confirmed by a decrease in HIF-1α protein as visualized by immunohistochemical staining of HCT116 xenografts.

The surgical orthotopic implantation (SOI) models have been used regard to drug sensitivity and metastasis as a bridge linking pre-clinical and clinical research in drug development [44]. In fact, LW6 exhibited inhibition of both migration and invasion of HCT116 cancer cells in *in vitro* migration and *in vitro* invasion assay respectively (data not shown). Therefore, *in vivo* metastasis

analysis can be carried out using orthotopic-transplant models of HCT116 colon cancer cells expressing GFP or luciferase and the inhibition of micrometastases by LW6 can be visualized.

In summary, LW6 promoted degradation of HIF-1α by up-regulating VHL expression. This study suggests that VHL may be an effective target for the development of a HIF-1 inhibitor, and LW6 can be developed as a HIF-1α inhibitor for cancer therapeutics.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bcp.2010.06.018.

References

[1] Semenza GL. Targeting HIF-1 for cancer therapy. Nat Rev Cancer 2003;3:721–32.
[2] Zhong H, De Marzo AM, Laughner E, Lim M, Hilton DA, Zagzag D, et al. Overexpression of hypoxia-inducible factor 1alpha in common human cancers and their metastases. Cancer Res 1999;59:5830–5.
[3] Semenza GL. Evaluation of HIF-1 inhibitors as anticancer agents. Drug Discov Today 2007;12:853–9.
[4] Pili R, Donehower RC. Is HIF-1 alpha a valid therapeutic target? J Natl Cancer Inst 2003;95:498–9.
[5] Eguchi H, Ikuta T, Tachibana T, Yoneda Y, Kawajiri K. A nuclear localization signal of human aryl hydrocarbon receptor nuclear translocator/hypoxia-inducible factor 1beta is a novel bipartite type recognized by the two components of nuclear pore-targeting complex. J Biol Chem 1997;272:17640–7.
[6] Pollenz RS, Sattler CA, Poland A. The aryl hydrocarbon receptor and aryl hydrocarbon receptor nuclear translocator protein show distinct subcellular localizations in Hepa 1c1c7 cells by immunofluorescence microscopy. Mol Pharmacol 1994;45:428–38.
[7] Seagroves T, Johnson RS. Two HIFs may be better than one. Cancer Cell 2002;1:211–3.
[8] Lal A, Peters H, St Croix B, Haroon ZA, Dewhirst MW, Strausberg RL, et al. Transcriptional response to hypoxia in human tumors. J Natl Cancer Inst 2001;93:1337–43.
[9] Kubo T, Maezawa N, Osada M, Katsumura S, Funae Y, Imaoka S, et al. an environmental endocrine-disrupting chemical, inhibits hypoxic response via degradation of hypoxia-inducible factor 1alpha (HIF-1alpha): structural requirement of bisphenol A for degradation of HIF-1alpha. Biochem Biophys Res Commun 2004;318:1006–11.
[10] Shin DH, Kim JH, Jung YJ, Kim KE, Jeong JM, Chun YS, et al. Preclinical evaluation of YC-1, a HIF inhibitor, for the prevention of tumor spreading. Cancer Lett 2007;255:107–16.
[11] Yeo EJ, Chun YS, Park JW. New anticancer strategies targeting HIF-1. Biochem Pharmacol 2004;68:1061–9.
[12] Rapisarda A, Uranchimeg B, Scudiero DA, Selby M, Sausville EA, Shoemaker RH, et al. Identification of small molecule inhibitors of hypoxia-inducible factor 1 transcriptional activation pathway. Cancer Res 2002;62:4316–24.
[13] Belozerov VE, Van Meir EG. Hypoxia inducible factor-1: a novel target for cancer therapy. Anti-cancer Drugs 2005;16:901–9.
[14] Jeong JW, Bae MK, Ahn MY, Kim SH, Sohn TK, Bae MH, et al. Regulation and destabilization of HIF-1alpha by ARD1-mediated acetylation. Cell 2002;111:709–20.
[15] Ivan M, Kondo K, Yang H, Kim W, Valiando J, Ohh M, et al. HIFalpha targeted for VHL-mediated destruction by proline hydroxylation: implications for O2 sensing. Science 2001;292:464–8.
[16] Jaakkola P, Mole DR, Tian YM, Wilson MI, Gielbert J, Gaskell SJ, et al. Targeting of HIF-alpha to the von Hippel-Lindau ubiquitilation complex by O2-regulated prolyl hydroxylation. Science 2001;292:468–72.
[17] Kallio PJ, Wilson WJ, O'Brien S, Makino Y, Poellinger L. Regulation of the hypoxia-inducible transcription factor 1alpha by the ubiquitin–proteasome pathway. J Biol Chem 1999;274:6519–25.
[18] Salceda S, Caro J. Hypoxia-inducible factor 1alpha (HIF-1alpha) protein is rapidly degraded by the ubiquitin–proteasome system under normoxic conditions. Its stabilization by hypoxia depends on redox-induced changes. J Biol Chem 1997;272:22642–7.
[19] Kaluzova M, Kaluz S, Lerman MI, Stanbridge EJ. DNA damage is a prerequisite for p53-mediated proteasomal degradation of HIF-1alpha in hypoxic cells and downregulation of the hypoxia marker carbonic anhydrase IX. Mol Cell Biol 2004;24:5757–66.
[20] Liu YV, Baek JH, Zhang H, Diez R, Cole RN, Semenza GL. RACK1 competes with HSP90 for binding to HIF-1alpha and is required for O(2)-independent and HSP90 inhibitor-induced degradation of HIF-1alpha. Mol Cell 2007;25:207–17.
[21] Lee K, Lee JH, Boovanahalli SK, Jin Y, Lee M, Jin X, et al. (Aryloxyacetylamino)benzoic acid analogues: a new class of hypoxia-inducible factor-1 inhibitors. J Med Chem 2007;50:1675–84.
[22] Kim DM, Chung KS, Choi SJ, Jung YJ, Park SK, Han GH, et al. RhoB induces apoptosis via direct interaction with TNFAIP1 in HeLa cells. Int J Cancer 2009;125:2520–7.
[23] Chung KS, Jang YJ, Kim NS, Park SY, Choi SJ, Kim JY, et al. Rapid screen of human genes for relevance to cancer using fission yeast. J Biomol Screen 2007;12:568–77.
[24] Nam JO, Kim JE, Jeong HW, Lee SJ, Lee BH, Choi JY, et al. Identification of the alphavbeta3 integrin-interacting motif of betaig-h3 and its anti-angiogenic effect. J Biol Chem 2003;278:25902–9.
[25] Kuo CJ, LaMontagne Jr KR, Garcia-Cardena G, Ackley BD, Kalman D, Park S, et al. Oligomerization-dependent regulation of motility and morphogenesis by the collagen XVIII NC1/endostatin domain. J Cell Biol 2001;152:1233–46.
[26] Won MS, Im N, Park S, Boovanahalli SK, Jin Y, Jin X, et al. A novel benzimidazole analogue inhibits the hypoxia-inducible factor (HIF)-1 pathway. Biochem Biophys Res Commun 2009;385:16–21.
[27] Cho H, Park H, Yang EG. A fluorescence polarization-based interaction assay for hypoxia-inducible factor prolyl hydroxylases. Biochem Biophys Res Commun 2005;337:275–80.
[28] Ravi R, Mookerjee B, Bhujwalla ZM, Sutter CH, Artemov D, Zeng Q, et al. Regulation of tumor angiogenesis by p53-induced degradation of hypoxia-inducible factor 1alpha. Genes Dev 2000;14:34–44.
[29] Puppo M, Battaglia F, Ottaviano C, Delfino S, Ribatti D, Varesio L, et al. Topotecan inhibits vascular endothelial growth factor production and angiogenic activity induced by hypoxia in human neuroblastoma by targeting hypoxia-inducible factor-1alpha and -2alpha. Mol Cancer Ther 2008;7:1974–84.
[30] Berchner-Pfannschmidt U, Tug S, Trinidad B, Oehme F, Yamac H, Wotzlaw C, et al. Nuclear oxygen sensing: induction of endogenous prolyl-hydroxylase 2 activity by hypoxia and nitric oxide. J Biol Chem 2008;283:31745–53.
[31] Berchner-Pfannschmidt U, Yamac H, Trinidad B, Fandrey J. Nitric oxide modulates oxygen sensing by hypoxia-inducible factor 1-dependent induction of prolyl hydroxylase 2. J Biol Chem 2007;282:1788–96.
[32] Kaelin Jr WG. Molecular basis of the VHL hereditary cancer syndrome. Nat Rev Cancer 2002;2:673–82.
[33] Los M, Jansen GH, Kaelin WG, Lips CJ, Blijham GH, Voest EE. Expression pattern of the von Hippel-Lindau protein in human tissues. Laboratory investigation. J Tech Methods Pathol 1996;75:231–8.
[34] Linehan WM, Vasselli J, Srinivasan R, Walther MM, Merino M, Choyke P, et al. Genetic basis of cancer of the kidney: disease-specific approaches to therapy. Clin Cancer Res 2004;10:6282S–9.
[35] Kapitsinou PP, Haase VH. The VHL tumor suppressor and HIF: insights from genetic studies in mice. Cell Death Diff 2008;15:650–9.
[36] Jung CR, Hwang KS, Yoo J, Cho WK, Kim JM, Kim WH, et al. E2-EPF UCP targets pVHL for degradation and associates with tumor growth and metastasis. Nat Med 2006;12:809–16.
[37] Akiyama H, Tanaka T, Itakura H, Kanai H, Maeno T, Doi H, et al. Inhibition of ocular angiogenesis by an adenovirus carrying the human von Hippel-Lindau tumor-suppressor gene in vivo. Invest Ophthalmol Visual Sci 2004;45:1289–96.
[38] Jones MK, Szabo IL, Kawanaka H, Husain SS, Tarnawski AS. von Hippel Lindau tumor suppressor and HIF-1alpha: new targets of NSAIDs inhibition of hypoxia-induced angiogenesis. FASEB J 2002;16:264–6.
[39] Kuroki T, Trapasso F, Yendamuri S, Matsuyama A, Alder H, Mori M, et al. Allele loss and promoter hypermethylation of VHL, RAR-beta RASSF1A, and FHIT tumor suppressor genes on chromosome 3p in esophageal squamous cell carcinoma. Cancer Res 2003;63:3724–8.
[40] Alleman WG, Tabios RL, Chandramouli GV, Aprelikova ON, Torres-Cabala C, Mendoza A, et al. The in vitro and in vivo effects of re-expressing methylated von Hippel-Lindau tumor suppressor gene in clear cell renal carcinoma with 5-aza-2'-deoxycytidine. Clin Cancer Res 2004;10:7011–21.
[41] Rapisarda A, Uranchimeg B, Sordet O, Pommier Y, Shoemaker RH, Melillo G. Topoisomerase I-mediated inhibition of hypoxia-inducible factor 1: mechanism and therapeutic implications. Cancer Res 2004;64:1475–82.
[42] Creighton-Gutteridge M, Cardellina 2nd JH, Stephen AG, Rapisarda A, Uranchimeg B, Hite K, et al. Cell type-specific, topoisomerase II-dependent inhibition of hypoxia-inducible factor-1alpha protein accumulation by NSC 644221. Clin Cancer Res 2007;13:1010–8.
[43] Zhou J, Schmid T, Frank R, Brune B. PI3K/Akt is required for heat shock proteins to protect hypoxia-inducible factor 1alpha from pVHL-independent degradation. J Biol Chem 2004;279:13506–13.
[44] Hoffman RM. Orthotopic metastatic mouse models for anticancer drug discovery and evaluation: a bridge to the clinic. Invest New Drugs 1999;17:343–59.