Emetine Promotes von Hippel-Lindau-Independent Degradation of Hypoxia-Inducible Factor-2α in Clear Cell Renal Carcinoma

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ABSTRACT

Inactivating mutations of the von Hippel-Lindau (VHL) tumor suppressor gene are associated with inherited VHL syndrome, which is characterized by susceptibility to a variety of neoplasms, including central nervous system hemangioblastoma and clear cell renal cell carcinoma (CCRCC). Mutations in the VHL gene are also found in the majority of sporadic clear cell renal carcinoma, the most common malignant neoplasm of the human kidney. Inactivation of VHL ubiquitin ligase is associated with normoxic stabilization of hypoxia-inducible factor-1α and 2-α (HIF-1α and HIF-2α), transcriptional regulators of tumor angiogenesis, invasion, survival, and glucose utilization. HIF-2α has been particularly implicated in the development of CCRCC. Although several inhibitors of HIF-1α have been described, these drugs typically have a minimal effect on HIF-2α. 786-O is a VHL-deficient CCRCC cell line that constitutively expresses only HIF-2α and is therefore suitable for the screening of novel HIF-2α inhibitors. Using this cell line, we have identified emetine as a specific inhibitor of HIF-2α protein stability and transcriptional activity. Without altering HIF-2α mRNA level, emetine rapidly and dramatically down-regulated HIF-2α protein expression in 786-O cells. HIF-2α down-regulation was accompanied by HIF-2α ubiquitination and was reversed by proteasome inhibition. Emetine-induced HIF-2α down-regulation was confirmed in three additional VHL-renal cancer cell lines, was insensitive to the prolyl hydroxylase inhibitor dimethyloxaloylglycine, and did not require neural precursor cell expressed developmentally down-regulated-8, suggesting that emetine accesses a previously undescribed cullin-independent proteasome degradation pathway for HIF-2α. These data support the use of emetine or structurally related compounds as useful leads for the identification of novel HIF-2α inhibitors.

Introduction

Inactivating mutations of the von Hippel-Lindau (VHL) tumor-suppressor gene are associated with inherited VHL syndrome, which is characterized by a variety of neoplasms, including central nervous system hemangioblastoma and clear cell renal cell carcinomas (CCRCCs) (Krieg et al., 2000; Rankin et al., 2006; Kaelin, 2009). Mutations in the VHL gene are also found in sporadic CCRCCs, the most common malignant neoplasm of the human kidney (Kaelin 2009). VHL inactivation results in the deregulated expression of HIF-1α and HIF-2α transcription factors (Krieg et al., 2000; Wiesener et al., 2001; Rankin et al., 2005, 2006; Block et al., 2007; Kaelin, 2009). Hypoxia-inducible factor (HIF) is a central regulator in tumor angiogenesis, invasion, cell survival, and glucose metabolism (Kaur et al., 2005; Semenza, 2010). Hypoxia-inducible HIF-1α and HIF-2α dimerize with constitutively expressed HIF-1β, also termed aryl hydrocarbon receptor nuclear translocator (ARNT), to activate the transcription of hypoxia-responsive genes. In normoxia, HIF-α proteins are

ABBREVIATIONS: VHL, von Hippel-Lindau; CCRCC, clear cell renal cell carcinoma; HIF, hypoxia-inducible factor; ARNT, aryl hydrocarbon receptor nuclear translocator; CREB, cAMP-response element-binding protein; VEGF, vascular endothelial growth factor; GLUT-1, glucose transporter-1; TGF-α, transforming growth factor-α; DMOG, dimethylxaloylglycine; NEDD8, neural precursor cell expressed developmentally down-regulated-8; ALLN, N-acetyl-Leu-Leu-norleucinal; ALLM, N-acetyl-l-leucyl-l-leucyl-l-methioninal; PCR, polymerase chain reaction; siRNA, small interfering RNA; RT-PCR, reverse transcriptase-polymerase chain reaction; ELISA, enzyme-linked immunosorbent assay; CHX, cycloheximide; PS-341, bortezomib; MG132, N-benzoyloxycarbonyl (Z)-Leu-Leu-leucinal.
rapidly degraded by proteasomes after their oxygen-dependent ubiquitination that is dependent on VHL recognition (Isaacs et al., 2002; Brahami-Horn et al., 2005; Semenza, 2009). VHL recognition requires hydroxylation of proline residues in HIF-α and HIF proline hydroxylases use molecular oxygen and 2-oxoglutarate as cosubstrates for this purpose (Sudarshan et al., 2007; Kaelin, 2009). In hypoxia, or in the case of VHL inactivation, HIF-α proteins are stabilized leading to transcriptional activation of genes whose promoter regions contain hypoxia response elements (Jung et al., 2003; Isaacs et al., 2004; Smaldone and Maranchie, 2009). These target genes include vascular endothelial growth factor (VEGF), glucose transporter-1 (GLUT-1), transforming growth factor-α (TGF-α), platelet-derived growth factor, and erythropoietin (Smaldone and Maranchie, 2009).

Even though HIF-2α, also termed endothelial PAS domain protein 1, is 48% identical in sequence to HIF-1α, and like HIF-1α is induced by hypoxia (Kaur et al., 2005) and stabilized by loss of VHL (Maxwell et al., 1999; Kaelin, 2009), HIF-1α and HIF-2α are believed to have distinct functions, both in normal cellular physiology and in tumorigenesis (Hu et al., 2003; Wang et al., 2005; Löfstedt et al., 2007; Rankin et al., 2008; Imamura et al., 2009). HIF-2α mRNA is abundantly expressed in lung, heart, liver, and other organs under normoxia, whereas HIF-1α mRNA is normally expressed at a much lower level (Krieg et al., 2000; Hu et al., 2003). HIF-1α and HIF-2α have been reported to have contrasting properties in the VHL-deficient CCRCC cell lines 786-O and RCC4, with HIF-1α retarding and HIF-2α enhancing the growth of tumor xenografts in mice (Raval et al., 2005). Likewise, two earlier studies showed that 786-O CCRCC cells stably expressing HIF-1α were not tumorigenic in mice, whereas forced expression of HIF-2α in the same background was tumorigenic (Kondo et al., 2002; Maranchie et al., 2002). In contrast, however, Imamura et al. reported that HIF-1α promoted the growth of colon cancer cells, whereas HIF-2α restrained their growth (Imamura et al., 2009). Taken together, these data suggest that the cellular function of both transcription factors is likely to be context-dependent.

Although several small-molecule inhibitors of HIF-1α have been described previously (Lin et al., 2004; Chau et al., 2005; Zhou et al., 2005; Choi et al., 2006; Faivre et al., 2006; Thomas et al., 2006), reports of HIF-2α inhibitors are less frequent. Because 786-O CCRCC cells express HIF-2α but not HIF-1α (Wiesener et al., 2001; Hu et al., 2003; Semenza 2003; Isaacs et al., 2004; Löfstedt et al., 2007), this cell line is useful for the identification of novel HIF-2α inhibitors.

Emetine is a protein synthesis inhibitor that blocks the translocation of peptidyl-tRNA from the acceptor site to the donor site on the ribosome (Grollman and Huang 1973). Emetine, extracted from Cephaelis ipecacuanha, has been used as a drug for the treatment of amoebiasis, as an antibacterial or antiviral agent, and as an emetic (Cushny 1918; Zhou et al., 2005). Emetine also has been evaluated in Phase II clinical studies for the treatment of solid tumors (Zhou et al., 2005). Recently, emetine was reported to specifically inhibit hypoxia-induced HIF-1α in breast tumor cells; however, its mechanism of action and possible impact on HIF-2α were not described previously (Zhou et al., 2005). In this study, we have examined the ability of emetine to inhibit HIF-2α in several VHL-CCRC cell lines, and we have investigated its mechanism of action.
bated with anti-HIF-2α antibody at 4°C for 16 h. Protein Gamma-Bind Plus Sepharose beads (GE Healthcare, Chalfont St. Giles, Buckinghamshire, UK) were added, incubated at 4°C for 2 h, and samples were processed for Western blot using anti-ubiquitin antibody as described.

Real-Time RT-PCR Analysis. Total mRNA was extracted using RNasy Mini Kit (Qiagen, Valencia, CA). Real-time RT-PCR was performed as described previously (Isaacs et al., 2005). In brief, cDNA was prepared using total RNA and the TaqMan Reverse Transcription Kit (Applied Biosystems, Foster City, CA). For reverse transcription, 200 ng of total RNA was used in 20 μl of reaction mixture containing 1× TaqMan RT buffer (TaqMan RT-PCR kit; Applied Biosystems), 5.5 mM magnesium chloride, 500 μM concentration of each dNTP, 2.5 μM random hexamer, 0.4 U/μl RNase inhibitor, and 1.25 U/μl Multiscribe Reverse Transcriptase. Reverse transcription was performed for 10 min at 25°C, 30 min at 48°C, and 5 min at 95°C using the PE9700 thermal cycler (Applied Biosystems). The reaction mixture was diluted to 100 μl of with Tris-EDTA. Five microliters of diluted cDNA was used for real-time PCR. Real-time PCR primers were designed using Primer Express software (Applied Biosystems). Analysis of mRNA expression was carried out using the ABI Prism 7700 Sequence Detection System (Applied Biosystems). Each 25 μl of amplification reaction contained 5 μl of sample, 5.5 μl of distilled H2O, 1 μl of each of 5 μM forward primer and reverse primer, and 12.5 μl of SYBR Green PCR master mix (Applied Biosystems). PCR cycling conditions were as follows: 10 min at 95°C, followed by 40 two-step cycles of 95°C for 15 s and 60°C for 60 s. Detection of the PCR product was monitored by measuring the increase in fluorescence caused by the binding of SYBR Green dye to double-stranded DNA. Emission spectra of all tubes were collected every cycle in the last 60 s of the primer annealing/elongation step in the ABI Prism7700 Sequence Detection System (Applied Biosystems). All samples were normalized to the level of 18S ribosomal RNA (rRNA). The number of PCR cycles to reach the fluorescence threshold value is the cycle threshold (Ct). Ct values for the control (18S rRNA) were determined, and relative RNA levels were calculated by the comparative Ct method as described by the manufacturer. Experiments were performed in duplicate.

VEGF and TGF-α ELISA. 786-O cells were seeded in 10-cm dishes 1 day before experiments. Serum-free medium and the indicated drug treatments were supplied to the cells for 24 h. Secreted VEGF level in conditioned media was determined using a Human VEGF Immunoassay kit (R&D Systems, Minneapolis, MN), and TGF-α level in total cell lysates was determined using Human TGF-α Immunoassay kit (R&D Systems) following the manufacturer’s instructions.

Results

Emetine Promotes VHL-Independent HIF-2α Down-Regulation. The terpenoid tetrahydroisoquinoline alkaloid emetine has been reported to inhibit hypoxic activation of HIF-1α in the breast tumor cell line T47D, although the mechanism responsible for this effect was not identified (Zhou et al., 2005). Emetine is a protein synthesis inhibitor and it has been explored previously as a chemotherapeutic agent (Cushny, 1918; Zhou et al., 2005). We sought to investigate whether emetine could also inhibit HIF-2α. We exposed 786-O CCRCC cells, which express HIF-2α under normoxia and are deficient in both HIF-1α and VHL (Maxwell et al., 1999; Kaelin 2009), to emetine and measured HIF-2α mRNA and protein expression. As shown in Fig. 1A (top), HIF-2α protein was highly expressed in untreated normoxic cells. Emetine dose-dependently down-regulated HIF-2α protein after 4 h of treatment, at concentrations as low as 0.05 μM. These results were confirmed in three additional CCRCC cell lines that also lack VHL expression (Fig. 1C). Because HIF-2α dimerization with ARNT can affect HIF-2α protein stability (Isaacs et al., 2004), we examined the possible impact of emetine on ARNT protein expression (Fig. 1A, middle). ARNT levels were not affected by emetine treatment. We next determined whether emetine might affect HIF-2α protein expression by inhibiting its synthesis. We treated 786-O cells with 0.5 to 5.0 μM cycloheximide (CHX) for 4 h to address this question. The impact of CHX on HIF-2α protein expression was much less dramatic than that of emetine (Fig. 1B). The data suggest that emetine-induced down-regulation of HIF-2α protein is not likely to involve protein synthesis inhibition. This hypothesis is supported by data obtained from pulsing cells briefly (30 min) with [35S]methionine to detect total protein synthesis. When 786-O cells were so treated during the final 30 min of a 4-h exposure to either emetine (0.1 μM) or CHX (1 μM), both drugs had a small but equivalent impact on HIF-2α protein synthesis.
on methionine incorporation, although neither drug caused toxicity at the concentrations and incubation times used (Fig. 1E). We also investigated whether emetine-stimulated down-regulation of HIF-2α might be mediated at the level of HIF-2α transcription. We measured HIF-2α mRNA by quantitative real-time RT-PCR, and we found that neither emetine nor CHX had any effect (Fig. 1D).

**Emetine-Induced HIF-2α Degradation Is Proteasome-Dependent.** To examine whether emetine-induced HIF-2α degradation required proteasome activity, we pretreated 786-O cells with the proteasome inhibitors PS-341, MG132, or ALLN before emetine addition. As shown in Fig. 2, A, B, and D, when we pretreated cells with three different proteasome inhibitors for 1 h and then treated with emetine for 4 h, HIF-2α protein in nuclear lysates of emetine-treated cells was protected from emetine. These data show that emetine, in the absence of VHL, nevertheless promotes degradation of HIF-2α in a proteasome-dependent manner. To further demonstrate that emetine-mediated HIF-2α degradation proceeds via the proteasome, we determined whether emetine influenced the intensity of HIF-2α ubiquitination in proteasome-inhibited 786-O cells. As shown in Fig. 2C, HIF-2α protein immunopre-

**Fig. 2.** Emetine-induced HIF-2α degradation is proteasome-dependent. A, 786-O cells were treated with indicated concentrations of the proteasome inhibitor, PS-341 (1-h pretreatment), 0.3 μM emetine, or with a combination of these agents, for 4 h. HIF-2α protein level in nuclear lysates was measured by Western blotting. Topoisomerase II (Topo II) expression is shown as a loading control. B, 786-O cells were treated with the proteasome inhibitor MG132 (10 μM, 1-h pretreatment), 0.3 μM emetine, or with a combination of these agents, for 4 h. HIF-2α and ARNT protein levels in nuclear lysates were measured by Western blotting. CREB expression is shown as a loading control. C, 786-O cells were treated with 100 nM PS-341, 0.3 μM emetine, or with a combination of these agents for 4 h. Protein lysates (1 mg) were immunoprecipitated with anti-HIF-2α antibody, and resultant blots were probed with an anti-ubiquitin antibody. After stripping, the membrane was reprobed with HIF-2α antibody. D, 786-O cells were pretreated with 100 μM ALLN for 1 h and then treated with 0.3 μM emetine for 4 h or with each agent separately. HIF-2α protein level in whole-cell lysates was measured by Western blotting; α-tubulin level is shown as loading control. E, 786-O cells were pretreated with 0.5 mM DMOG for 1 h and then exposed to 0.3 μM emetine for 4 h. HIF-2α protein was measured by Western blotting; α-tubulin is shown as loading control. F and G, cells were pretreated for 1 h with 100 μM ALLM (F) or 1 μM calpastatin peptide (G) and then were treated and analyzed as in E; α-tubulin is shown as a loading control.
cipitated from cells treated for 4 h with both emetine and a proteasome inhibitor was highly ubiquitinated compared with the other treatment groups. Although it is proteasome-mediated, emetine-induced HIF-2α degradation does not require HIF proline hydroxylation. Treatment of cells with the prolyl hydroxylase inhibitor DMOG did not block emetine-induced HIF-2α degradation (Fig. 2E). Thus, unlike VHL, the E3 ligase responsible for targeting HIF-2α to the proteasome in emetine-treated cells does not require HIF hydroxylation as a recognition motif. Finally, emetine-induced degradation of HIF-2α was not blocked by either calpain inhibitor II (ALLM) or the calpain inhibitory peptide calpastatin (Fig. 2, F and G).

**Emetine-Induced HIF-2α Degradation Does Not Require Cullin Neddylation.** Oxygen-dependent degradation of HIF-1α and HIF-2α requires HIF-α recognition by VHL, leading to ubiquitination by the VHL, Cullin, and Elongin B/C E3 ubiquitin ligase complex (Sufan et al., 2004; Tan et al., 2008). Activation of cullin-containing E3 ubiquitin ligases requires covalent modification of a conserved lysine residue in the cullin protein by the ubiquitin-like protein NEDD8 (Chew and Hagen, 2007). In the VHL, Cullin, and Elongin B/C complex, Elongin C bridges VHL to Cul2 and Cul2 associates with elongin C, NEDD8, and Rbx1 (Sufan et al., 2004). Although it does not require VHL for its activity, we next

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**Fig. 3.** Cullin neddylation is not required for emetine-induced HIF-2α degradation. 786-O cells were transfected with either control siRNA or NEDD8 siRNA. A, to confirm NEDD8 silencing, NEDD8 mRNA was analyzed by quantitative RT-PCR 72 h after transfection. Statistical significance was calculated using SigmaPlot 9.0 (Systat Software Inc., San Jose, CA). Each bar represents the mean ± S.D. (n = 3). B, 786-O cells were transfected with control or NEDD8 siRNA as in A, and after 72 h, the cells were treated with 0.3 μM emetine for an additional 4 h. HIF-2α protein level was assessed in nuclear lysates by Western blotting. CREB expression is shown as a loading control.

**Fig. 4.** Emetine-mediated inhibition of HIF-dependent transcription. A, 786-O cells were untreated or exposed to the indicated concentrations of emetine for 4 h, and mRNA expression for GLUT-1 and VEGF were analyzed by quantitative RT-PCR. Results shown are representative of three separate experiments. B and C, 786-O cells were untreated or exposed to the indicated concentrations of emetine or CHX for 24 h in serum-free medium. The conditioned media were subjected to a VEGF ELISA (B), and TGF-α protein was measured in whole-cell lysates with a TGF-α ELISA (C). Statistical significance was calculated using SigmaPlot 9.0 (Systat Software Inc., San Jose, CA). Each bar represents the mean ± S.D. (n = 3).
asked whether emetine-induced HIF-2α degradation requires cullin neddylation. We transfected 786-O cells with NEDD8 (or control) siRNA. After 72 h (to allow for silencing of NEDD8), we treated cells with emetine for an additional 4 h. The level of NEDD8 mRNA was decreased by approximately 90% in NEDD8-silenced cells (Fig. 3A). However, NEDD8 knockdown had no impact on the ability of emetine to efficiently down-regulate HIF-2α protein (Fig. 3B). These data suggest that emetine-induced HIF-2α ubiquitination, unlike HIF-1α ubiquitination mediated by VHL, does not use a cullin-containing E3 ligase.

**HIF-2α-Mediated Transcription Is Inhibited by Emetine.** GLUT-1, VEGF, and TGF-α are HIF target genes in CCRCC (Smaldone and Maranchie 2009). We monitored GLUT-1 and VEGF mRNA levels in 786-O cells after exposure to emetine and found the transcription of both genes to be significantly and dose-dependently reduced by emetine (Fig. 4A). To investigate this phenomenon further at the protein level, we treated 786-O cells with the indicated concentrations of either emetine or CHX for 24 h, and then we determined the amount of secreted VEGF protein in the conditioned media. Emetine, but not CHX, caused a significant dose-dependent decrease in secreted VEGF protein (Fig. 4B). Treatment with 1.0 μM emetine resulted in a 75% decrease of secreted VEGF protein compared with untreated cells. TGF-α protein expression in cell lysate was similarly inhibited by emetine but not by cycloheximide (Fig. 4C). These data show that emetine-induced HIF-2α protein degradation is accompanied by the inhibition of HIF-2α-dependent transcription.

**Discussion**

786-O CCRCC cells are VHL-deficient and constitutively express HIF-2α but not HIF-1α. Therefore, these cells provide an excellent platform to screen for VHL- and oxygen-independent inhibitors of HIF-2α (Wiesener et al., 2001; Hu et al., 2003; Semenza, 2003; Isaacs et al., 2004). In this study, we have demonstrated that emetine is such an inhibitor, affecting both HIF-2α protein stability and its transcriptional regulation of endogenous target genes. We confirmed these findings using three additional HIF-2α-expressing, VHL-deficient CCRCC cell lines. Emetine has been reported previously to inhibit hypoxia-induced HIF-1α activation in breast tumor cells (Zhou et al., 2005). We confirmed that emetine reduced HIF-1α expression in several cancer cell lines, including MCF7 (VHL wild-type breast cancer), PC3 (VHL wild-type prostate cancer), and UMRC2 cells (VHL-deficient CCRCC that expresses HIF-1α) (data not shown). Thus, emetine possesses the relatively unique property of inhibiting both HIF-1α and HIF-2α expression independently of VHL.

Although HIF-2α inhibition by emetine is oxygen- and VHL-independent, it is accomplished by proteasome-mediated degradation that is preceded by enhanced HIF-2α ubiquitination. Although we have not identified the E3 ubiquitin ligase responsible for emetine-induced HIF-2α ubiquitination, it is unlikely to be a cullin-containing complex (as is the VHL complex) because these E3 ligases require the covalent modification of a conserved lysine residue in the cullin protein with the ubiquitin-like protein NEDD8. We found that NEDD8 molecular silencing had no effect on emetine-induced down-regulation of HIF-2α protein in 786-O cells.

HIF prolyl hydroxylation is necessary to create the recognition motif for VHL binding (Sudarshan et al., 2007; Kaelin, 2009; Semenza, 2009). The calcium-dependent protease calpain has also been reported to regulate HIF-1α stability under certain circumstances (Zhou et al., 2006). However, we found that emetine-mediated degradation of HIF-2α was calpain-independent because two different calpain inhibitors failed to block emetine-induced HIF-2α depletion. Furthermore, the emetine effect on HIF-2α was also insensitive to the prolyl hydroxylase inhibitor DMOG, offering additional support to the hypothesis that emetine accesses a previously undescribed calcium-dependent proteasome degradation pathway that is distinct from the one mediated by VHL.

By down-regulating HIF-2α protein, emetine affected the transcription of several endogenous HIF-dependent genes in 786-O cells. Both GLUT-1 and VEGF mRNA expression were reduced after emetine treatment, as was the synthesis of VEGF and TGF-α proteins. These gene products are essential HIF-regulated proangiogenic and pro-survival proteins that mediate CCRCC tumorigenicity. Thus, our data support further medicinal chemistry investigation of emetine as a useful platform for the identification of novel HIF-2α inhibitors.

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**References**


