Molecular mechanisms for the activity of PX-478, an antitumor inhibitor of the hypoxia-inducible factor-1α

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Abstract

We have reported previously that PX-478 (*S*-2-amino-3-[4′-*N*,*N*,-bis(chloroethyl)amino]phenyl propionic acid *N*-oxide dihydrochloride) has potent antitumor activity against a variety of human tumor xenografts associated with the levels of the hypoxia-inducible factor-1α (HIF-1α) within the tumor. We now report that PX-478 inhibits HIF-1α protein levels and transactivation in a variety of cancer cell lines. Hypoxia-induced vascular endothelial growth factor formation was inhibited by PX-478, whereas baseline levels of vascular endothelial growth factor in normoxia were unaffected. Studies of the mechanism of PX-478 action showed that HIF-1α inhibition occurs in both normoxia and hypoxia and does not require pVHL or p53. In addition, PX-478 decreases levels of HIF-1α mRNA and inhibits translation as determined by ^{35}S labeling experiments and reporter assays using the 5′ untranslated region of HIF-1α. Moreover, to a lesser extent, PX-478 also inhibits HIF-1α deubiquitination resulting in increased levels of polyubiquitinated HIF-1α. The inhibitory effect of PX-478 on HIF-1α levels is primarily due to its inhibition of translation because HIF-1α translation continues in hypoxia when translation of most proteins is decreased. We conclude that PX-478 inhibits HIF-1α at multiple levels that together or individually may contribute to its antitumor activity against HIF-1α-expressing tumors. [Mol Cancer Ther 2008;7(1):90–100]

Introduction

Hypoxia (0.05-5% O_2) is a feature of solid tumors, from the smallest tumor a few millimeters in diameter to the very largest tumor (1). Constitutive hypoxia occurs because the tumor continually outgrows its blood supply (2) and because oxygen is constantly metabolized by tumor cells and cannot diffuse more than 100 to 180 μm from the capillaries (3). There is also perfusion-related hypoxia, which is secondary to cyclic, transient cessation of blood flow in the structurally and functionally abnormal tumor blood vessels (4). Severe hypoxia (<0.02% O_2) results from chronic occlusion of tumor blood vessels due to pressure from the tumor or obstruction by tumor cells, leading to necrosis (5).

Cells adapt to hypoxia by down-regulating oxygen- and energy-dependent processes, such as mRNA translation or protein synthesis (6), while simultaneously up-regulating specific genes that promote angiogenesis and stress survival. This process is regulated at the initiation stages, that is, the assembly of the m7-GTP cap binding eIF-4E/eIF-4A/eIF-4G (eIF-4F) and 40S ribosome binding eIF-2/GTP/met-tRNA ternary complexes (7). One protein whose translation is maintained during hypoxia is the hypoxia-inducible factor 1α (HIF-1α), the oxygen-regulated component of the HIF-1 heterodimer, a transcription factor required for adaptation to hypoxia. HIF-1α translation is also increased by activation of the phosphatidylinositol 3-kinase/Akt signaling pathway (8) through activation of mammalian target for rapamycin (9).

HIF-1α can also be regulated at the protein level by the oxygen-dependent hydroxylation of HIF-1α by specific prolyl hydroxylases (PHD1-PHD3). This results in the binding of the von Hippel-Lindau protein (pVHL) E3 ubiquitin ligase complex (10) leading to HIF-1α ubiquitination and proteasomal degradation (11). When oxygen concentrations become limiting during hypoxia, HIF-1α is stabilized and translocates to the nucleus where it heterodimerizes with HIF-1β and activates transcription of downstream target genes. Other regulators of HIF-1α include human double minute 2 (also known as MDM2), which is recruited to HIF-1α by p53 resulting in human double minute 2–mediated HIF-1α ubiquitination and proteasomal degradation (12), and heat shock protein 90, which binds to HIF-1α to prevent its degradation (13).

Increased HIF-1 activity increases tumor vascularization and energy metabolism, whereas loss of that activity dramatically suppresses these responses (14–16). Clinically, increased tumor HIF-1α is a marker of aggressive disease, associated with poor patient prognosis and treatment failure for a number of cancers, including breast, lung, ovarian, cervical, esophageal, and oropharyngeal cancers (17–19). Activation of HIF-1 also occurs in relatively few normal tissues, for example, in skin during wound healing (20) and in diseases, such as age-related macular degeneration (21). Taken together, the experimental and clinical evidence suggest that inhibitors of HIF-1α may provide a new approach to cancer therapy (22, 23).

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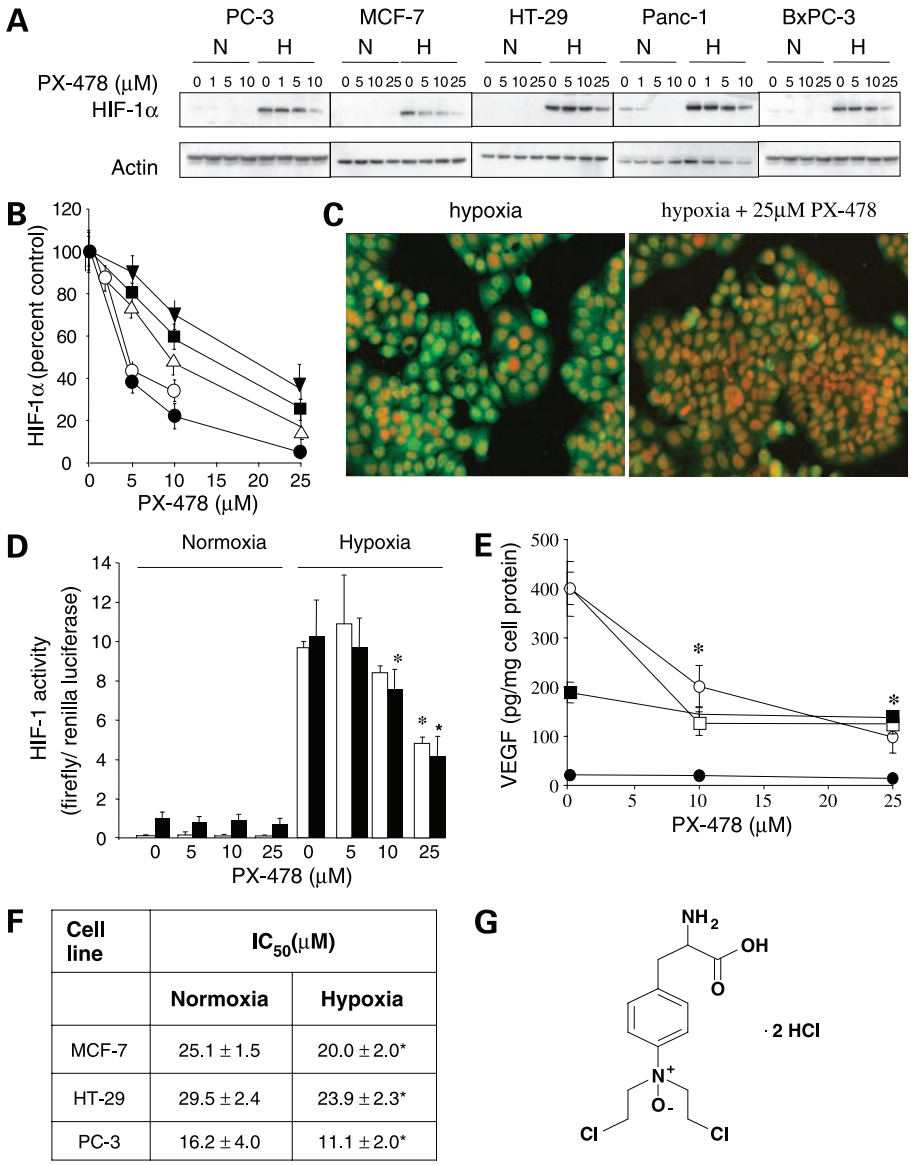
We have reported previously the antitumor activity of an experimental HIF-1α inhibitor, PX-478 (*S*-2-amino-3-[4′-*N*,*N*,-bis(chloroethyl)amino]phenyl propionic acid *N*-oxide dihydrochloride; Fig. 1G), against a variety of human tumor xenografts in immunodeficient mice where antitumor activity was positively correlated with the levels of HIF-1α (24). PX-478 was initially identified through a screen for compounds that lowered cellular HIF-1α levels.



Cell line	IC_{50}(μM)	
	Normoxia	Hypoxia
MCF-7	25.1 ± 1.5	20.0 ± 2.0*
HT-29	29.5 ± 2.4	23.9 ± 2.3*
PC-3	16.2 ± 4.0	11.1 ± 2.0*

Figure 1. PX-478 decreases HIF-1α protein levels and inhibits HIF-1 transactivating activity and the expression of downstream genes. **A,** MCF-7, HT-29, PC-3, Panc-1, and BxPC-3 cells were treated with PX-478 for 16 h under normoxic (air, 5% CO_2) or hypoxic (1% O_2, 5% CO_2, 94% N_2) conditions. Cell lysates were prepared and HIF-1α protein levels were measured by Western blotting. Typical blots with actin as a loading control. **B,** blots were quantitated by densitometry relative to actin: (●) MCF-7 cells, (▼) HT-29 cells, and (○) PC-3 cells, (△) Panc-1 cells, and (■) BxPC-3 cells. *Points,* mean of three experiments; *bars,* SE. **C,** HT-29 cells were exposed to hypoxia for 16 h with or without 25 μmol/L PX-478, fixed, and permeabilized. HIF-1α protein was measured by immunofluorescence staining (*green*). Nuclei were stained with 4′,6-diamidino-2-phenylindole (*orange*). **D,** MCF-7 cells (*open columns*) and HT-29 cells (filled *columns*) were transiently transfected with firefly luciferase under the control of the phosphoglycerate kinase hypoxia-responsive element reporter or control constitutive *Renilla* luciferase then exposed to normoxia or hypoxia for 16 h in the presence of PX-478 as indicated. Luciferase activity was expressed as the ratio of firefly to *Renilla* luciferase. *Columns,* mean of three experiments; *bars,* SE. *, $P < 0.05$, compared with the appropriate control without PX-478. **E,** VEGF protein per milligram cellular protein in total cell lysates (●, ○) or that secreted into the medium (■, □) in normoxia (●, ■) or hypoxia (○, □) as measured by ELISA after 16-h treatment of MCF-7 cells with PX-478. *Points,* mean of three experiments; *bars,* SE. *, $P < 0.05$, compared with control without PX-478. **F,** cell growth inhibition by PX-478. MCF-7, HT-29, and PC-3 cells were treated for 16 h with varying concentrations of PX-478 in air or hypoxia. The cells were washed free of drug and grown in air for a further 56 h. Cell growth was then assessed by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay. Mean of three experiments carried out in duplicate; *bars,* SE. *, $P < 0.05$, compared with the IC_{50} under aerobic conditions. **G,** structure of PX-478.

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PX-478 causes marked tumor regression, prolonged delays in tumor growth, and cure of some animals. PX-478 has recently entered phase I clinical trial in patients with advanced cancer.

We now report the cellular pharmacology of PX-478 and show that it lowers HIF-1α protein levels independently of oxygen, pVHL, or p53, thus decreasing HIF-1 transactivating activity and downstream gene expression. Mechanisms that may account for lowering of HIF-1α protein by PX-478 are primarily through inhibition of HIF-1α translation and, to a lesser extent, decreasing levels of HIF-1α mRNA and inhibiting HIF-1α deubiquitination. We propose that inhibition of translation by PX-478 accounts for the selectivity for HIF-1α because HIF-1α translation is maintained in hypoxia, whereas translation of most proteins is inhibited.

Materials and Methods

Cells and Reagents

PC-3, MCF-7, HT-29, Panc-1, and BxPC-3 cells were obtained from American Type Culture Collection. Human RCC4 and RCC4/VHL cells were obtained from Dr. P. Ratcliffe (Welcome Trust Center for Human Genetics). Human colon carcinoma cells expressing wild-type p53 ($HCT116^{+/+}$) and $HCT116^{-/-}$ cells from which p53 has been deleted were from Dr. Bert Vogelstein (Johns Hopkins University). HIF-1α antibody was from BD Transduction Labs, whereas lamin A/C, actin, and hemagglutinnin (HA) antibodies were from Santa Cruz Biotechnology. PX-478 was obtained from ProlX Pharmaceuticals, and actinomycin D and cycloheximide were from Sigma.

Hypoxia Assay

Culture flasks were incubated for various times at 37°C in humidified air, 5% CO_2 (normoxia) or 1% O_2, 5% CO_2, 94% N_2 (hypoxia) using an InVivo Hypoxia Workstation 400 with a Ruskin hypoxic gas mixer (Biotrace International). After incubation, cells were washed twice with cold PBS equilibrated with the same gas mixture. In some studies, medium was removed at the end of incubation and stored at 80°C for measurement of vascular endothelial growth factor (VEGF) levels.

Cell Viability Assay

Cells were grown in normoxia or hypoxia for 16 h with or without PX-478, washed thrice with warm DMEM to remove the drug, and then grown in normoxia for a further 56 h before measuring cell viability by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay.

VEGF ELISA

About 10^7 cells were lysed in 200 μL lysis buffer [150 mmol/L NaCl, 50 mmol/L Tris (pH 7.5), 1 mmol/L phenylmethylsulfonyl fluoride, 2 μg/mL aprotinin, 2 μg/mL leupeptin, 0.1 mmol/L sodium orthovanadate, 1% NP40, 0.2% SDS]. The lysate was clarified and an aliquot of supernatant was retained for protein measurement using the Bio-Rad Protein Detection System. Human VEGF in cell lysates and secreted VEGF were determined using an ELISA measuring VEGF165 and VEGF121 isoforms (R&D Systems).

Western Blotting

Western blotting was done as described previously (25). Blots were quantified using ImageQuant software (Molecular Dynamics). Nuclear and cytoplasmic extracts were prepared using NE-PER Nuclear and Cytoplasmic Extraction Reagents (Pierce Biotechnology).

Immunoprecipitation and Deubiquitination Assays

Immunoprecipitation was done by incubating 1 mg cell lysate protein with 2 μg HIF-1α antibody in lysis buffer and protein A Sepharose beads (Sigma-Aldrich) overnight at 4°C. The assay for deubiquitination was carried out as described previously (26). To prepare polyubiquitinated HIF-1α, MCF-7 cells were transfected with HA-ubiquitin using LipofectAMINE 2000 (Invitrogen) and left to recover overnight. Cells were then exposed to hypoxia in the presence of 5 μmol/L MG132 and then exposed to normoxia for a further 1 h to allow accumulation of polyubiquitinated HIF-1α. To prepare cell lysates as a source of deubiquitinating activity, MCF-7 cells were grown to 70% confluency then placed in hypoxia for 16 h after which cells were harvested. For PX-478 treatment, lysates were incubated for 15 min on ice with 5 μmol/L MG132 or PX-478 as indicated. Assays were done by adding 50 μL cell lysate (3 mg/mL) to 50 μL of a 25% slurry of HIF-1α substrate bound to protein A Sepharose beads and incubated at 37°C for 1 h after which samples were placed on ice. Beads were washed and samples were eluted by boiling in sample buffer and analyzed by SDS-PAGE using anti-HA antibody.

Immunofluorescent Staining

Cells were grown on chamberslides (BD) for 24 h then fixed in 4% formaldehyde/PBS for 20 min followed by permeabilization in 0.2% Triton X-100/PBS for 10 min and reactive aldehyde groups quenched in 100 mmol/L glycine/100 mmol/L lysine/PBS for 30 min. RNA was digested with 10 μg/mL RNase, DNase-free (Roche Applied Science) for 30 min at 37°C, and coverslips were blocked for 30 min in 10% goat serum/PBS. Primary anti-HIF-1α monoclonal antibody or monoclonal isotype control antibody (Dako) was incubated for 24 h at 4°C in 5% goat serum/PBS followed by 1-h incubation with 5 μg/mL Alexa Fluor-488 goat anti-mouse antibody (Invitrogen) and an additional 1 h with 5 μg/mL Alexa Fluor 488 donkey anti-goat antibody and a 1:2,000 dilution of BOBO3 (Invitrogen). Slides were coverslipped with Prolong mounting medium (Invitrogen) and imaged on a Nikon TE300 fluorescence microscope and CoolSNAP digital camera (RS Photometrics).

HIF-1α mRNA Measurement

Total RNA was isolated from MCF-7 cells using the PARIS kit (Ambion, Applied Biosystems) according to the manufacturer's protocol. TaqMan quantitative reverse transcription-PCR was done on the ABI 7300 system using the TaqMan One-Step reverse transcription-PCR Master Mix kit and predesigned primer/probe pairs for HIF-1α and β_2-microglobulin (Applied Biosystems). Normalization and analyses were carried out with β_2-microglobulin using as the internal reference by the -CT method (27) using the Applied Biosystems GeneAmp 5700 SDS software.

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Reporter Assays

A pGL3 firefly luciferase reporter plasmid containing the phosphoglycerate kinase hypoxia-responsive element was supplied by Dr. Ian Stratford (University of Manchester). The empty pGL3 control plasmid and the pRL-CMV *Renilla* luciferase plasmid were obtained from Promega. Cells were transfected with HIF-1 reporter plasmid, or pGL3 control plasmid, and pRL-CMV *Renilla* luciferase plasmid using LipoTAXI (Stratagene) and 24 h later were exposed to hypoxia for 16 h with or without 25 μmol/L PX-478. Firefly and *Renilla* luciferase activities were measured using the Dual Luciferase Reporter Assay System (Promega). Monocistronic vectors containing the 5′-untranslated region (UTR) of HIF-1α and c-Myc as well as a control firefly luciferase (28) were kindly provided by Dr. G.J. Goodall (Institute of Medical and Veterinary Science). MCF-7 cells were transfected using LipofectAMINE 2000 and treated as described above for the HIF reporter plasmid above.

^{35}S Protein Synthesis

MCF-7 breast or HT-29 colon cancer cells were grown for 16 h in air or 1% O_2 with or without 25 μmol/L PX-478. The cells were then washed with cysteine/methionine-free DMEM containing 10% fetal bovine serum dialyzed against the same medium and incubated with 0.2 mCi/mL [^{35}S]cysteine and [^{35}S]methionine (Pro-Mix L-^{35}S cell labeling mix, Amersham Radiochemicals) in the same medium. For the hypoxic cells, the washing and incubation media were preequilibrated in hypoxia, and all manipulations were carried out in the hypoxia chamber. Cells were harvested by washing in PBS at 4°C, and total cell lysates were prepared in lysis buffer as above and analyzed by SDS-PAGE and autoradiography.

Results

PX-478 Decreases Nuclear HIF-1α Protein and Inhibits HIF-1 Activity

PX-478 was identified previously through a screen for compounds that lowered cellular HIF-1α levels. Validation studies confirmed that PX-478 inhibits the hypoxia-induced increase in HIF-1α protein (Fig. 1A), with IC_{50} (mean ± SE; n = 3) for PC-3 prostate cancer cells of 3.9 ± 2.0 mol/L, MCF-7 breast cancer cells of 4.0 ± 2.0 mol/L, HT-29 colon cancer cells of 19.4 ± 5.0 mol/L, Panc-1 pancreatic cancer cells of 10.1 ± 1.9 mol/L, and BxPC-3 pancreatic cancer cells of 15.3 ± 4.8 mol/L (Fig. 1B). PX-478 also decreased the low but measurable HIF-1α protein levels observed in PC-3 prostate and Panc-1 pancreatic cancer cells under normoxic conditions with IC_{50} of 2.5 ± 1.2 and 3.2 ± 1.2 μmol/L, respectively. In contrast, PX-478 treatment did not affect the levels of HIF-1β protein under either normoxic or hypoxic conditions (data not shown), suggesting that PX-478 exhibits some specificity for HIF-1α. Using cell fractionation procedures, we found that PX-478 treatment of HT-29 cells under hypoxic conditions resulted in decreased HIF-1α levels in both nuclear and cytosolic fractions, suggesting that PX-478 does not affect the nuclear import of the HIF-1α in hypoxic cells (data not shown).

Immunofluorescence staining of PX-478-treated HT-29 cells also showed no difference in the subcellular localization of HIF-1α, whereas overall HIF-1α staining was decreased (Fig. 1C). Consistent with the decrease in HIF-1α protein levels, PX-478 treatment also significantly decreased the activity of a HIF-1 reporter construct transiently transfected into MCF-7 and HT-29 cells with IC_{50} of ~25 μmol/L (Fig. 1D), confirming that the PX-478-mediated decrease in HIF-1α protein levels results in decreased HIF-1 transactivation activity. Additionally, PX-478 treatment significantly decreased the hypoxia-induced expression of VEGF, a HIF-1-regulated protein, in MCF-7 cells in a dose-dependent manner but did not affect basal levels of VEGF formation under normoxic conditions (Fig. 1E). Similar results were obtained with HT-29 cells (data not shown). Furthermore, PX-478 caused a small although significantly greater inhibition of cell growth under hypoxic compared with normoxic conditions, with hypoxia/normoxia IC_{50} ratio of 1.25 for MCF-7 cells, 1.20 for HT-29 cells, and 1.45 for PC-3 cells (Fig. 1F). Because the *in vivo* antitumor activity of PX-478 is mediated in part through inhibition of HIF-1-dependent VEGF formation and angiogenesis, PX-478 treatment would not be expected to show major differences in *in vitro* cytotoxicity in hypoxia compared with normoxia.

Thus, the results show that PX-478 lowers HIF-1α protein levels and HIF-1 transactivation in hypoxia and, when expressed, in normoxia but does not affect the subcellular distribution of HIF-1α. There was not a marked difference in the cytotoxicity of PX-478 under normoxic and hypoxic conditions, as the consequences of HIF-1 inhibition by PX-478 are primarily seen on angiogenesis, which accounts for its *in vivo* antitumor activity.

Time Course of PX-478 Effects on HIF-1α Protein and Its Specificity

To observe the kinetics of the effects of PX-478 on HIF-1α levels, we monitored HIF-1α induction in the absence or presence of PX-478 in MCF-7 cells over time and found that although HIF-1α was detectable about 30 min after transfer to the hypoxia work station, it took 6 to 8 h of hypoxia for HIF-1α to reach maximal levels in MCF-7 cells. Intriguingly, PX-478 did not begin to inhibit HIF-1α levels until after 8 h hypoxia but showed almost complete inhibition by 16 h (Fig. 2A and B). When PX-478 was removed from the medium, HIF-1α returned to pretreatment levels within 4 h (Fig. 2C). Western blotting showed very small or no effect of PX-478 treatment on the levels of other proteins in HT-29 cells after 16 h, including heat shock protein 90, the heat shock protein 90 client proteins AKT, c-Src, and Raf-1, as well as other proteins whose degradation is controlled by ubiquitination, cyclin B1, histone H1, and mutant p53 (Fig. 2D). Raf-1 levels were decreased by PX-478 in normoxia, but not hypoxia, whereas cyclin B1 levels were increased by PX-478 in hypoxia but not normoxia.

Thus, PX-478 appears to have a delayed effect in inhibiting hypoxia-induced HIF-1α with a more rapid recovery on its removal.

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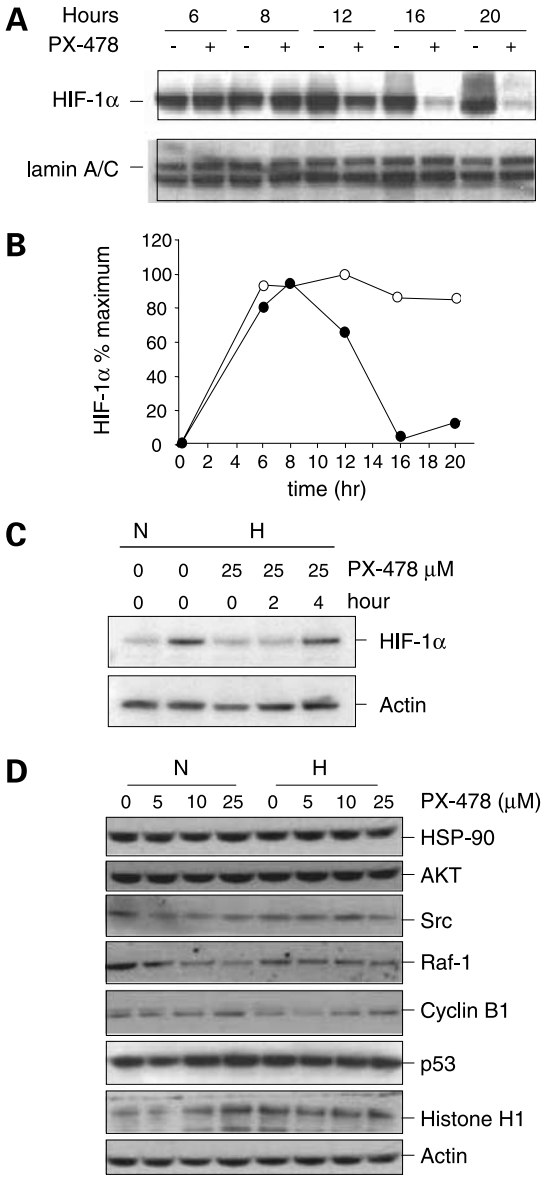

Figure 2. Time course and specificity of PX-478. **A,** MCF-7 cells were incubated under hypoxic (1% O_2, 5% CO_2, 94% N_2) conditions together with 25 μmol/L PX-478 for the times indicated. Nuclear extracts were prepared at various times and HIF-1α was measured by Western blotting. Typical blot with lamin A/C as a loading control. **B,** quantitation of the HIF-1α blot by densitometry expressed relative to lamin A/C: control MCF-7 cells (○) and MCF-7 cells treated with 25 μmol/L PX-478 (●). **C,** MCF-7 cells were exposed to hypoxia (*H*) for 16 h with or without 25 μmol/L PX-478. Drug was removed, the cells were washed with hypoxic medium (time 0), and the recovery of nuclear HIF-1α levels under hypoxic conditions at 2 and 4 h were measured by Western blotting. **D,** effect of PX-478 treatment on expression levels of a panel of proteins. HT-29 cells were exposed to PX-478 in air or hypoxia for 16 h. Total cell extracts were prepared and protein levels were measured by Western blotting.

PX-478 Inhibition of HIF-1α Does Not Require pVHL or p53

The major mechanism for the rapid physiologic regulation of HIF-1α protein is through pVHL via the ubiquitin-proteasomal pathway, which results in the oxygen-dependent proteasomal degradation of HIF-1α. To investigate the effects of PX-478 on this pathway, we used human RCC4 renal carcinoma cells lacking functional pVHL and the corresponding cell line (RCC4/VHL) in which functional pVHL is stably expressed. The RCC4 cells, consistent with the loss of pVHL, show elevated levels of HIF-1α protein under normoxic conditions and no significant difference between HIF-1α levels in normoxia and hypoxia (Fig. 3A). In contrast, the RCC4/VHL cells have little detectable HIF-1α in normoxia but show a 10-fold increase in HIF-1α levels in hypoxia by densitometry (Fig. 3A). PX-478 treatment decreased HIF-1α levels in both RCC4 and RCC4/VHL cells under hypoxic conditions with IC_{50} (±SE) of

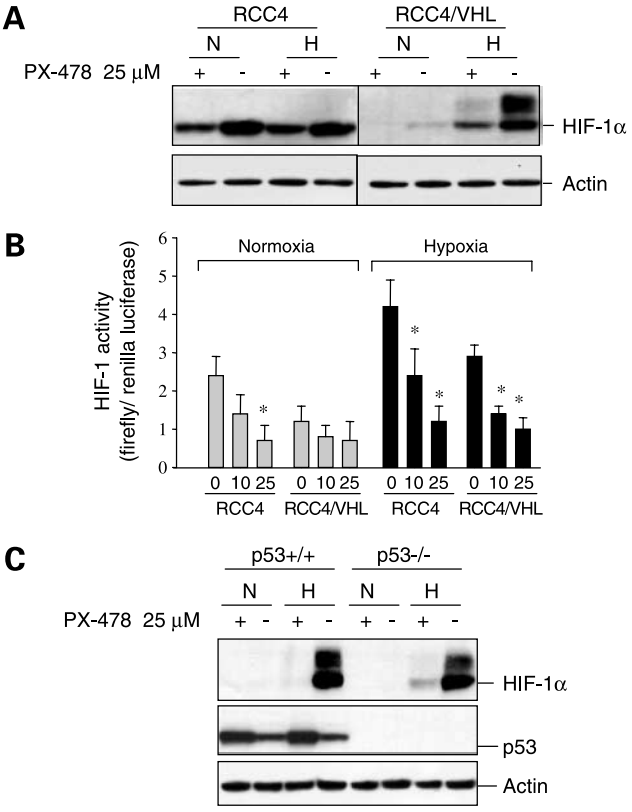

Figure 3. PX-478 decreases HIF-1 independently of pVHL or p53 pathways. **A,** human renal cancer RCC4 cells lacking pVHL and RCC4/VHL cells into which pVHL has been reintroduced were exposed to 25 μmol/L PX-478 under normoxic (*N*; air, 5% CO_2) or hypoxic (*H*; 1% O_2, 5% CO_2, 94% N_2) conditions for 16 h. Cell extracts were prepared and HIF-1α levels were measured by Western blotting. **B,** RCC4 and RCC4/VHL cells were transiently transfected with firefly luciferase under the control of the phosphoglycerate kinase hypoxia-responsive element reporter or control constitutive *Renilla* luciferase then exposed to normoxia or hypoxia for 16 h in the presence of 25 μmol/L PX-478 as indicated. Luciferase activity was expressed as the ratio of firefly to *Renilla* luciferase. Mean of three experiments; bars, SE. *, $P < 0.05$, compared with the appropriate control without PX-478. **C,** human $HCT116^{+/+}$ colon cancer cells expressing wild-type p53 and $HCT116^{-/-}$ cells without p53 were exposed to normoxic or hypoxic conditions with and without 25 μmol/L PX-478 for 16 h. Cell extracts were prepared and HIF-1α and p53 levels were measured by Western blotting.

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6.9 ± 1.9 and 13.5 ± 1.3 mol/L, respectively, and 5.1 ± 2.0 mol/L in RCC4 cells under normoxic conditions. This corresponds to a 35% and 45% decrease in HIF-1α levels in hypoxic RCC4 and RCC4/VHL cells, respectively, and a 25% decrease in normoxic HIF-1α levels in RCC4 cells as determined by densitometry. HIF-1 transactivating activity in both RCC4 and RCC4/VHL cells was also significantly inhibited by treatment with PX-478 (Fig. 3B). To investigate the effect of p53 on hypoxia-induced HIF-1α accumulation, we used HCT116 human colon carcinoma cells either expressing wild-type p53 or HCT116 cells from which p53 was deleted (Fig. 3C). PX-478 treatment decreased HIF-1α protein in both cell lines. We also observed an increase in p53 levels by PX-478 in HCT116 wild-type cells in both normoxia and hypoxia, which was also observed in the HT-29 cells in normoxia (Fig. 2D), possibly reflecting cell-specific differences in the cellular response to PX-478. Hence, the results show that the effect of PX-478 in decreasing HIF-1α is independent of both pVHL and p53.

PX-478 Modulates HIF-1α Ubiquitination

To determine if the PX-478-induced decrease in HIF-1α is mediated by the proteasomal degradation pathway, we transfected MCF-7 cells with HA-tagged ubiquitin before their exposure to hypoxia and PX-478 in the absence or presence of MG132, an inhibitor of the 26S proteasome. Treatment with 25 and 50 μmol/L PX-478 resulted in a 42% and 58% decrease in HIF-1α levels, respectively, compared with untreated cells, whereas treatment with MG132 before exposure to 25 and 50 μmol/L PX-478 resulted in a 20% and 25% decrease in HIF-1α levels, respectively, compared with cells treated with MG132 alone (Fig. 4A). Hence, MG132 attenuates but does not completely prevent the PX-478-induced decrease in HIF-1α. Increasing the concentration of MG132 also could not completely block the PX-478-induced decrease in HIF-1α (data not shown), suggesting that the mechanism for HIF-1α lowering occurs in part independently of the proteasomal degradation pathway. Intriguingly, we also observed that immunoprecipitated HIF-1α was highly ubiquitinated in a PX-478 concentration-dependent manner in the treated cells when compared with the untreated control (Fig. 4B). This could be seen in both MG132-treated and untreated hypoxic MCF-7 cells but was more apparent in the presence of MG132 when approximately equal amounts of HIF-1α protein were loaded on the Western blot (Fig. 4C).

PX-478 could possibly alter levels of ubiquitinated HIF-1α in two ways: by increasing HIF-1α ubiquitination or by decreasing its deubiquitination. To determine the mechanism by which PX-478 increases levels of ubiquitinated HIF-1α, HA-ubiquitinated HIF-1α conjugated to protein A Sepharose beads was incubated for 1 h at 37°C with lysates from cells exposed to PX-478 under hypoxic conditions or untreated normoxic cell lysate to which PX-478 was added (Fig. 4D). PX-478 added to either the untreated cell lysates or cell lysates from PX-478-treated cells showed increased levels of ubiquitinated HIF-1α in a concentration-dependent manner. Because HA-ubiquitin was not transfected into the cell lysates used, the change observed in HIF-1α after PX-478 treatment must have been caused by decreased deubiquitination rather than increased ubiquitination. To put these changes into context, we measured the effect of PX-478 on the rate of HIF-1α degradation in MCF-7 cells under hypoxic conditions in the presence of cycloheximide to block new HIF-1α translation (Fig. 4E). We observed a 40% decrease in HIF-1α levels 1.5 h following cycloheximide treatment in PX-478-treated cells compared with cells not treated with PX-478. However, there was no difference in HIF-1α levels between PX-478-treated and untreated cells after 3-h incubation with cycloheximide. This suggests that although there was an initial increase in the rate of HIF-1α degradation caused by PX-478, there was no overall difference in the rate of HIF-1α degradation between PX-478-treated and untreated cells after 3 h exposure to cycloheximide, suggesting that PX-478 does not increase the overall rate of HIF-1α degradation.

Taken together, the results show that HIF-1α ubiquitination and degradation still occur under hypoxic conditions and that PX-478 inhibits the deubiquitination of ubiquitinated HIF-1α. This results in an initial increase in HIF-1α degradation through the 26S proteasome but does not affect the overall rate of HIF-1α degradation. Furthermore, proteasomal inhibition is unable to block to PX-478-induced decrease in HIF-1α, suggesting that the PX-478-mediated inhibition of HIF-1α deubiquitination cannot account for the previously observed decrease in HIF-1α levels.

PX-478 Inhibits HIF-1α Transcription

To determine the level at which PX-478 mediates its effect, we first investigated its effect on HIF-1α mRNA levels in MCF-7 cells. We found that treatment with 12 and 25 μmol/L PX-478 for 16 h significantly decreased the levels of HIF-1α mRNA in both normoxia and hypoxia (Fig. 5A) but did not affect the levels of β_2-microglobulin mRNA, a component of the MHC class I molecule that was used as a normalization control. However, levels of genes not known to be regulated by hypoxia or HIF-1, such as Ste-20-related kinase (SPAK) and squamous cell carcinoma antigen recognized by T cells (SART1), also showed some PX-478-induced decrease in normoxia but no change in hypoxia (data not shown), suggesting that PX-478 does exhibit some nonspecific effects. Hence, we show that PX-478 treatment decreases HIF-1α mRNA levels in both normoxia and hypoxia.

PX-478 Inhibits HIF-1α Translation

Next, we determined the effect of PX-478 on HIF-1α translation by comparing the rate of HIF-1α synthesis in PX-478-treated or untreated cells. MCF-7 cells were exposed to PX-478 for 16 h (in normoxia) then cultured in hypoxia-equilibrated medium, and the rate of increase in nuclear HIF-1α protein was measured by Western blotting. We found that the rate of HIF-1α synthesis was markedly lower in cells treated with PX-478 than in cells that were not treated with PX-478 (Fig. 5B and C). This suggests that although PX-478 lowers HIF-1α mRNA levels as described above, it also inhibits the rate of synthesis of HIF-1α.

To address the effect of PX-478 on translation, global cellular protein synthesis was measured by [^{35}S]cysteine/

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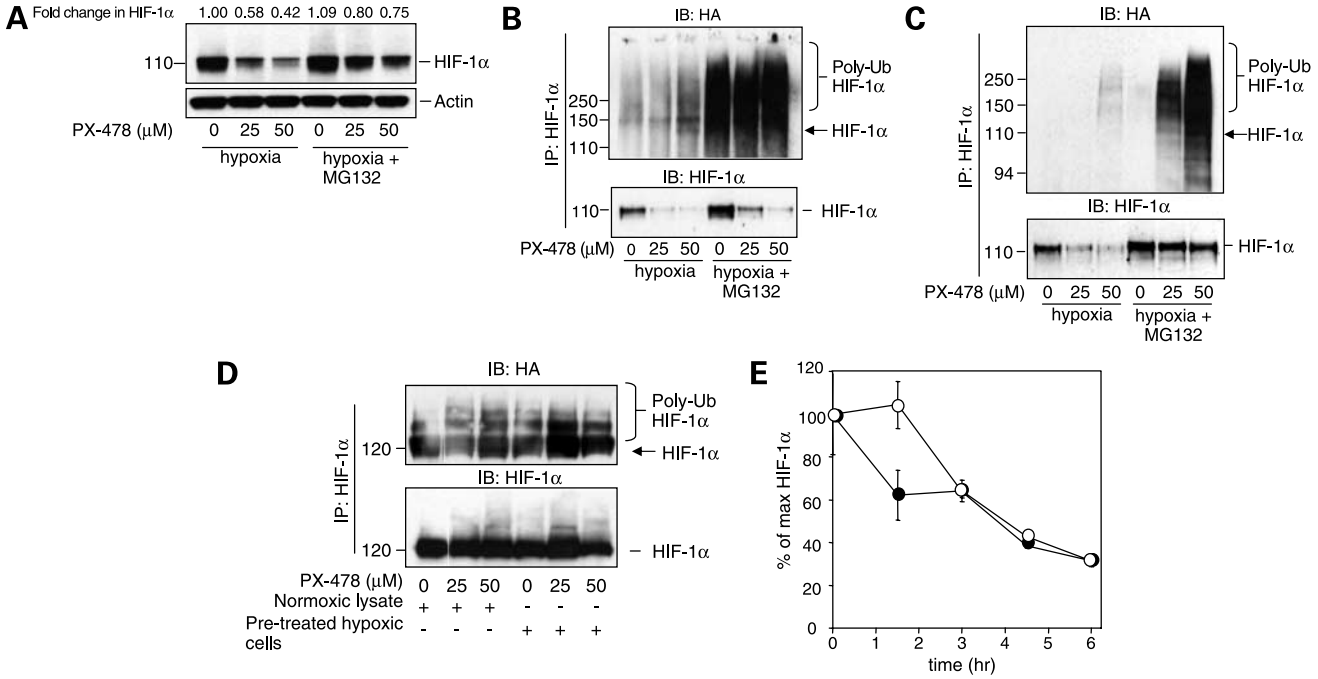

Figure 4. A minor role for PX-478 in the proteasomal-dependent degradation of HIF-1α. **A,** effect of PX-478 on HIF-1α ubiquitination. MCF-7 cells were transiently transfected with HA-tagged ubiquitin (*Ub*) and 24 h later incubated for 16 h in hypoxia with or without 25 μmol/L PX-478 with or without MG132 (5 μmol/L). Total cell lysates were analyzed by Western blotting using anti-HA and anti-HIF-1α antibody. Actin was used as a loading control. HIF-1α band intensities normalized to actin analyzed by densitometry are shown above each lane. Normalized values were divided by values obtained in untreated samples in hypoxia. **B,** HIF-1α was immunoprecipitated from 1 mg of the MCF-7 lysate in **A** and analyzed by Western blotting using anti-HA and anti-HIF-1α antibody. **C,** Western blots of the same lysate from **B**, where loading was adjusted to give approximately the same amount of HIF-1α. **D,** HIF-1α deubiquitination assay. HIF-1α polyubiquitinated with HA-ubiquitin was immunoprecipitated from MG132-treated HT-29 cells and incubated in air at 37°C for 1 h with untreated MCF-7 cell lysate and PX-478 at the concentrations shown or with lysate from MCF-7 cells exposed to hypoxia and PX-478 at the concentrations shown for 16 h. Beads were pelleted and immunoblotted with anti-HA to detect ubiquitin-HIF-1α or with anti-HIF-1α to detect total HIF-1α. **E,** MCF-7 cells were exposed to hypoxia for 10 h with (●) or without (○) 25 mol/L PX-478 and the levels of total cellular HIF-1α under hypoxic conditions were measured by Western blotting in the presence of cycloheximide to block translation. Mean of three determinations; *bars,* SE.

methionine incorporation where we found, as expected, that [^{35}S]cysteine/methionine incorporation after 20- and 40-min incubation was decreased during hypoxia in MCF-7 cells (Fig. 6A). However, [^{35}S]cysteine/methionine incorporation in normoxia and hypoxia in untreated cells was comparable after 60-min incubation, which may indicate saturation. After 20 min, PX-478 treatment resulted in a 32% inhibition of protein translation in normoxia and 36% in hypoxia although overall translation was inhibited by 27% in hypoxia (Fig. 6B). However, by 60 min, PX-478 inhibited 61% of overall protein translation in hypoxia, considerably more than the 13% inhibition of overall protein translation observed in normoxia, showing that PX-478 treatment inhibits the hypoxia-dependent component of total cellular protein synthesis but does not affect the rate of total cellular protein synthesis in normoxia. Similar results were obtained in HT-29 colon cancer cells (data not shown).

To study the mechanism of translation inhibition by PX-478, we examined the phosphorylation of eIF-2α and 4E-BP, which control the initiation of protein translation (29). Exposure of cells to hypoxia resulted in increased eIF-2α phosphorylation and decreased 4E-BP phosphorylation (Fig. 6C) as described previously (30). However, PX-478 treatment had no effect on the phosphorylation of eIF-2α or on 4E-BP dephosphorylation in hypoxia, indicating that PX-478 does not interfere with the known repressors of translation in hypoxia.

To investigate whether PX-478 inhibits HIF-1α translation specifically, we used reporter constructs in which the 5′-UTR from human HIF-1α, whose translation is known to be maintained in hypoxia, or the 5′-UTR of c-Myc, which is not known to play a role in the response to hypoxia, were inserted upstream of the firefly luciferase reporter (28, 31). These reporter plasmids or a control reporter plasmid under the control of a constitutively active promoter were transfected into MCF-7 cells, and the levels of firefly luciferase were measured and normalized to levels of total cellular protein assuming equal transfection efficiencies. Consequently, we show that PX-478 treatment results in a 30% and 55% inhibition of HIF-1α translation in normoxia and hypoxia, respectively (Fig. 6D). We also observed a 30% inhibition of c-Myc UTR-driven translation by PX-478 in both normoxia and hypoxia.

Taken together, the results show that PX-478 decreases global protein translation in normoxia and hypoxia but has a more pronounced effect on translation of proteins, such as HIF-1α in hypoxia.

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Discussion

We have reported previously the antitumor activity of the HIF-1α inhibitor, PX-478, against a variety of human tumor xenografts in immunodeficient mice (24). We now report the cellular pharmacology of PX-478 and possible mechanisms by which PX-478 is able to lower HIF-1α levels. PX-478 decreases hypoxia-induced HIF-1α protein levels in a panel of cancer cell lines and also decreases levels of constitutively elevated HIF-1α in Panc-1 pancreatic cancer and PC-3 prostate cancer, where elevated HIF-1α is due to increased phosphatidylinositol 3-kinase/Akt signaling (8) and HIF-1α gene amplification (32), and in RCC4 renal cell cancer, where elevated HIF-1α is due to loss of pVHL (33). PX-478 also lowers HIF-1α levels in isogenic HCT116 $p53^{+/+}$ and HCT116 $p53^{-/-}$ cells. Thus, the decrease in HIF-1α induced by PX-478 does not require oxygen, pVHL, or p53.

The decrease in HIF-1α protein levels is associated with a decrease in HIF-1 transactivating activity and in the decreased expression of one of the downstream targets of HIF-1, VEGF. Only hypoxia-mediated VEGF expression was decreased by PX-478 treatment, whereas normoxic VEGF expression, which is known to be dependent on other transcription factors, such as SP-1 (34), was not inhibited. We found that the effect of PX-478 in lowering HIF-1α levels was not immediate, taking about 8 h to develop. This might be explained by the metabolism of the PX-478 to an active constituent or to a slowly developing effect of PX-478. Hypoxia had minimal effect on the cytotoxicity of PX-478, which is perhaps unsurprising, because HIF-1α is a stress survival factor and cultured cells, even under hypoxic conditions, are minimally stressed.

We examined several other proteins and did not find PX-478 to cause a major change in their levels. PX-478 has some effect in increasing p53, in lowering Raf-1, on the transcription of *SPAK* and *SART1* and the translation of c-Myc in normoxia. Thus, whereas PX-478 may not be completely specific for lowering HIF-1α, of the proteins we examined, this is its major activity that appears to be responsible for its antitumor effects (24).

Hypoxia leads to a general down-regulation of protein translation by promoting Ser^{51} phosphorylation on eIF-2α and inhibition of eIF-4E through 4E-BP dephosphorylation (6, 29, 35, 36). PX-478 treatment did not affect the levels of phospho-eIF-2α or phospho-4E-BP, suggesting that PX-478 does not affect the hypoxia-dependent repression of translation. Although general protein synthesis is repressed during hypoxia as a survival mechanism, the synthesis of a small number of stress-responsive proteins, including

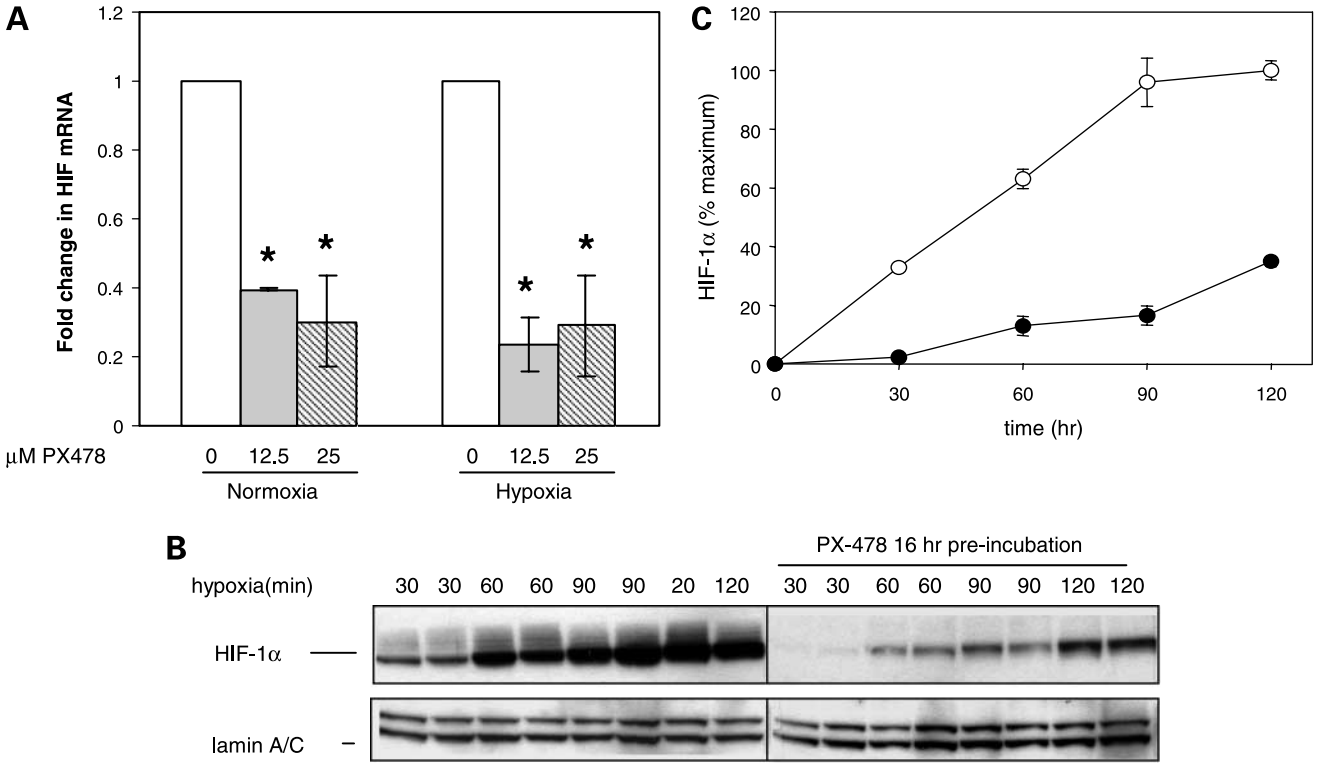

Figure 5. PX-478 decreases mRNA levels and inhibits synthesis of HIF-1α. **A,** quantitative reverse transcription-PCR of MCF-7 cells treated with 12.5 and 25 μmol/L PX-478 for 16 h in normoxia and hypoxia. HIF-1α transcript levels were measured by Taqman and normalized to levels of β_2-microglobulin. Average of three experiments; *bars,* S.E. *, $P \leq 0.05$, compared with values without drug. **B,** PX-478 inhibits the rate of synthesis of HIF-1α. Hypoxic medium was added to MCF-7 cells pretreated with 25 μmol/L PX-478 for 16 h and the rate of increase in nuclear HIF-1α protein was measured by Western blotting and compared with untreated cells. Experiments were done in duplicate with lamin A/C as a loading control. **C,** quantitation of the Western blot in **B** showing HIF-1α levels normalized to lamin A/C in PX-478 untreated (○) or treated (●) MCF-7 cells exposed to hypoxia.

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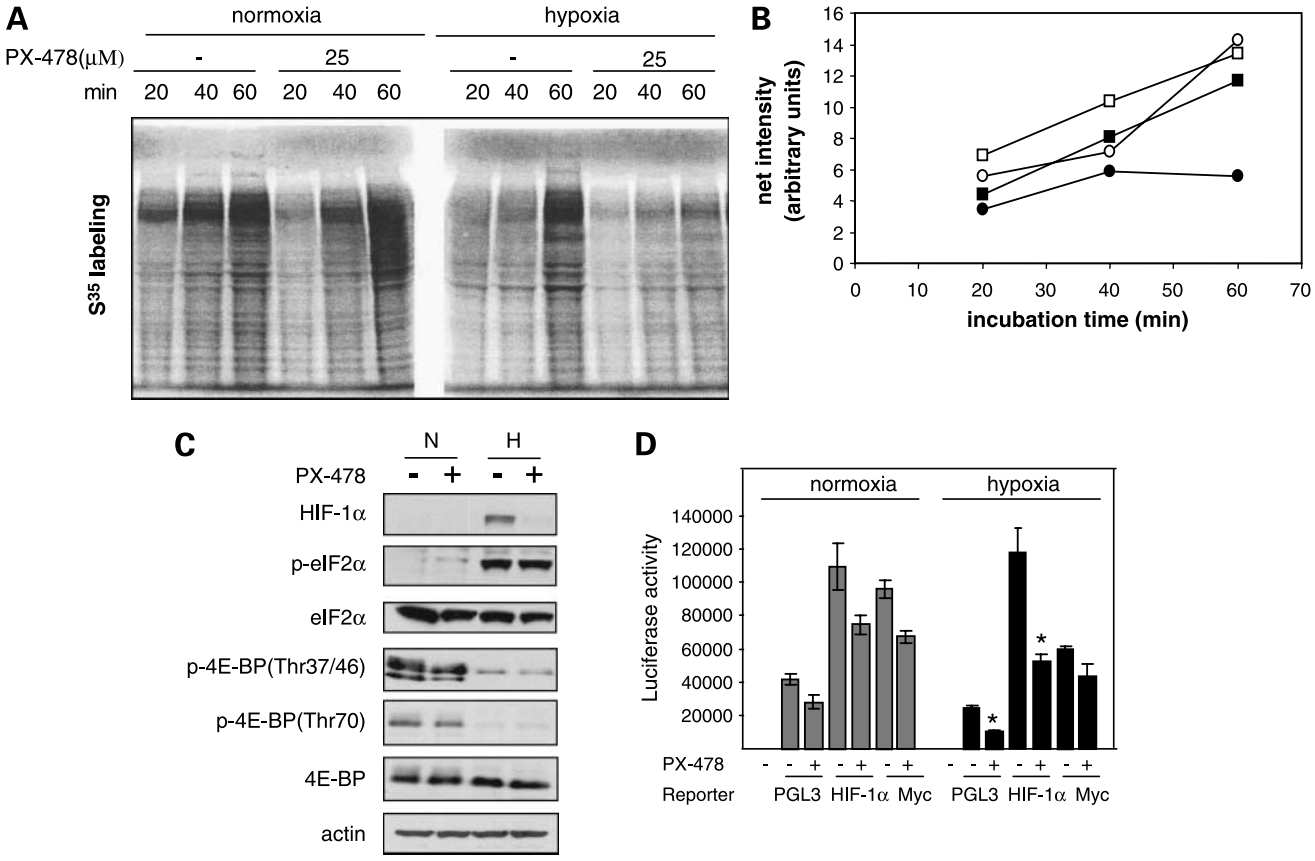

Figure 6. PX-478 inhibits HIF-1α translation. **A,** SDS-PAGE and autoradiography of total protein from cell lysates of MCF-7 cells grown for 16 h in normoxia or hypoxia with or without 25 μmol/L PX-478 and then with 0.2 mCi/mL [^{35}S]cysteine and [^{35}S]methionine for the indicated times. **B,** quantitation of each lane of the autoradiograph for untreated (□) or PX-478-treated (■) cells in normoxia or untreated (○) or PX-478-treated (●) cells in hypoxia. **C,** MCF-7 cells were incubated for 16 h under normoxic or hypoxic conditions with or without 25 μmol/L PX-478, lysed, and subjected to Western blotting for components of the protein translation regulatory pathway. **D,** MCF-7 cells were transfected with firefly luciferase vectors containing the 5′-UTRs of HIF-1α or c-Myc or with control firefly luciferase. The cells were then exposed to normoxia or hypoxia for 16 h with or without 25 μmol/L PX-478 and lysed, and luciferase activity was determined and normalized to cellular protein levels. Mean of four determinations; *bars,* SD. *, $P \leq 0.05$, compared with appropriate HIF-1α and c-Myc value without drug.

HIF-1α, is sustained through a process believed to be mediated by sites in the UTRs of these proteins that allow maintenance of translation under hypoxia (7). Using UTR reporter vectors to study the effects of PX-478 on HIF-1α and c-Myc translation (31), we found that HIF-1α UTR-driven translation was maintained during hypoxia but inhibited by PX-478, whereas c-Myc UTR translation was inhibited in hypoxia and showed only slight inhibition by PX-478. Thus, although PX-478 inhibits global translation in hypoxia, HIF-1α is the major protein inhibited by PX-478 probably because it is one of few proteins whose translation is maintained during hypoxia.

We also found that PX-478 treatment inhibited HIF-1α deubiquitination, thus increasing the levels of polyubiquitinated HIF-1α. HIF-1α deubiquitination may be mediated by the ubiquitin-specific protease 20 (37) but whether inhibition of ubiquitin-specific protease 20 is the mechanism by which PX-478 treatment decreases HIF-1α deubiquitination remains to be determined. One might expect that an increase in polyubiquitinated HIF-1α by PX-478, whatever the mechanism, would lead to an increase in proteasomal degradation. However, we have no direct evidence for this and the contribution of proteasomal degradation to lowering of HIF-1α levels by PX-478 under the conditions of our study may be minor. However, in other conditions, it could achieve greater importance.

Consequently, we found that PX-478 inhibits HIF-1α at multiple levels. PX-478 treatment resulted in decreased levels of HIF-1α mRNA, decreased translation of HIF-1α protein during both normoxia and hypoxia, and increased deubiquitination of HIF-1α. It is possible that the cumulative effects of PX-478 on the levels of HIF-1α transcription, translation, and ubiquitination may provide some specificity of PX-478 towards HIF-1α.

Furthermore, whereas the translation of most proteins is down-regulated in hypoxia, translation of HIF-1α is maintained. Hence, inhibition of HIF-1α translation in hypoxia by PX-478 is more pronounced than for other proteins.

Several other agents have been reported to lower HIF-1α protein levels, including topoisomerase I and II inhibitors

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(38, 39), phosphatidylinositol 3-kinase inhibitors (40), heat shock protein 90 inhibitors (41), microtubule cytoskeleton disrupting agents (42), HIV protease inhibitors (43), and YC-1, an activator of soluble guanyl cyclase that stimulates prolyl hydroxylase activity (44). None of these agents is selective for HIF-1α and the contribution of HIF-1α inhibition to their activity has not been established. In contrast, among the proteins we examined, PX-478 appears to be selective for lowering HIF-1α and is the only agent clearly shown to act at multiple levels through inhibition of both HIF-1α transcription and translation.

In summary, we have shown that PX-478 inhibits HIF-1α protein levels and HIF-1 transactivating activity in a variety of cancer cell lines. The inhibition occurs in both normoxic and hypoxic conditions and does not require pVHL, or p53. Three mechanisms were identified as contributing to the decrease in HIF-1α levels by PX-478: (*a*) inhibition of HIF-1α deubiquitination, which in the model we used appears to play a minor role and (*b*) reduction in HIF-1α mRNA levels together with (*c*) inhibition of HIF-1α translation play the major roles. The changes may help explain the potent antitumor activity of PX-478 against HIF-1α-expressing tumors.

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Molecular mechanisms for the activity of PX-478, an antitumor inhibitor of the hypoxia-inducible factor-1 α

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