Novel Thioredoxin Inhibitors Paradoxically Increase Hypoxia-Inducible Factor-α Expression but Decrease Functional Transcriptional Activity, DNA Binding, and Degradation

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Abstract

Purpose: Hypoxia-inducible factor-α (HIF-α) is a transcription factor that regulates the response to hypoxia. HIF-α protein is found at high levels in many cancers, and the redox protein thioredoxin-1 (Trx-1) increases both aerobic and hypoxia-induced HIF-α. Therefore, Trx-1 and HIF-α are attractive molecular targets for novel cancer therapeutics.

Experimental Design: We investigated whether two novel anticancer drugs AJM290 and AW464 (quinols), which inhibit Trx-1 function, can inhibit the HIF pathway.

Results: Treatment of several cancer cell lines with AJM290 or AW464 prevented the hypoxia-induced increase of vascular endothelial growth factor (VEGF) at subtoxic concentrations. AJM290 and AW464 also decreased VEGF in pVHL mutant renal cell carcinoma cells that constitutively overexpress HIF-α protein. They surprisingly up-regulated HIF-α expression in breast cancer cell lines in normoxia and hypoxia as well as in pVHL mutant cells. In the MDA-MB-468 breast cancer cell line, the compounds inhibited RNA and protein expression of the HIF-α target genes, carbonic anhydrase IX, VEGF, and BNIP3, concordantly with HIF-α up-regulation. Both compounds specifically inhibited HIF-α-dependent induction of hypoxia regulatory element-luciferase and HIF-1α hypoxia regulatory element-DNA binding. To analyze the HIF-1α domain inhibited by AJM290, we transfected cells with plasmids expressing a fusion protein of Gal linked to HIF-1α or HIF-1α COOH-terminal transactivation domain (CAD) with a Gal4-responsive luciferase reporter gene. AJM290 inhibited both the full-length HIF-1α and HIF-1α CAD transcriptional activity.

Conclusions: AJM290 and AW464 are inhibitors of HIF-1α CAD transcription activity and DNA binding, but they also inhibit degradation of HIF, in contrast to other Trx inhibitors.

Hypoxia-inducible factor (HIF) is an α,β heterodimeric transcription factor that directs a broad range of responses in hypoxic cells (1). Both proteins are members of the basic helix-loop-helix superfamily of transcription factors in which the basic helix-loop-helix domains bind to DNA (2). HIF-1α is a constitutive nuclear localized subunit that binds to available HIF-α (3). To date, three HIF-α isoforms have been described, with the best characterized being HIF-1α and HIF-2α. In the presence of oxygen, two prolyl sites within a central degradation domain of HIF-α are hydroxylated by a set of closely related Fe²⁺ and 2-OG-dependent dioxygenases (PHD1-3), which leads to HIF-α degradation via the pVHL E3 ubiquitin ligase complex and the 26S proteasome (4). Limiting oxygen levels or the availability of Fe³⁺ with iron chelators (5) allows HIF-α to escape proteolysis. In the nucleus, HIF α,β heterodimer interacts with coactivators, such CBP/p300, and becomes transcriptionally active (6). On activation, the HIF-αβ complex binds to target genes at sites containing the core recognition sequence 5’-RCGTG-3’, also known as the hypoxia regulatory element (HRE; ref. 7), which finally leads to up-regulation of genes involved in angiogenesis, glucose metabolism, and pH regulation (1).

The HIF transcription cascade has been shown to contribute to tumor progression and metastasis and plays an important part in the malignant phenotype (8–10). HIF-α is found at increased levels in a wide variety of human primary tumors compared with corresponding normal tissue and increases angiogenesis and other properties that promote increased vascularity and tumor progression (8–10). Besides physiologic hypoxia, genetic abnormalities frequently detected in human cancers, which include key oncogenes (HER-2, FRAP, H-RAS, and SRC) and tumor suppressor genes (pVHL, p53, and PTEN), are also associated with induction of HIF-α activity and expression of HIF-α-inducible genes (11–15).
Due to the involvement of HIF-α in tumor progression and angiogenesis, HIF-α is a promising molecular target for development of cancer therapeutics (16–18). Thioredoxin-1 (Trx-1), which is a ubiquitously expressed small redox protein with a conserved catalytic site (19), has been shown to regulate the activity of enzymes, such as apoptosis signal-regulating kinase-1 and protein kinases C α, β, ε, and ζ in a redox-dependent manner (20, 21). Trx-1 also increases the DNA binding of redox-sensitive transcription factors, which includes nuclear factor-κB and p53 (22, 23). It has been recently reported that increased expression of Trx-1 in cancer cells increases HIF-α protein levels and transactivating activity under both normoxic and hypoxic conditions (24). Trx-1 expression is highly expressed in several human primary cancers, including colon, cervix, lung, pancreatic, liver, colorectal, and squamous cell cancers (25–30).

Several inhibitors of Trx pathway have been developed. PX-12 (Trx-1 inhibitor) and pleurotin and PX478 (Trx-1 reductase inhibitors) have already been shown to downregulate hypoxia-induced increase and constitutive expression of HIF-1α and HIF-1α transcription factor activity (31, 32). Here, we investigated the effects of novel Trx-1 inhibitors AJM290 (indole-substituted quinol) and AW464 (benzothiazole-substituted quinol) on HIF-1α and its downstream targets (Fig. 1A). Both compounds have been shown to have in vitro antitumor activity against colon, renal, and breast cancer cell lines and in vivo antitumor activity in mice bearing breast, colon, and renal xenografts (33–36).

**Materials and Methods**

**Cell culture and materials.** Human breast cancer cell lines MDA-MB-468 and MDA-MB-231 and melanoma cell line MDA-MB-435 were maintained in DMEM. The pVHL-deficient RCCA and 786-0 renal carcinoma cell lines and their counterpart containing a stably transfected pVHL gene were cultured in α-MEM and DMEM, respectively, and maintained in selection with 500 µg/mL G418. Both α-MEM and DMEM were supplemented with 10% fetal bovine serum, 2 mmol/L l-glutamine, 50 IU/mL penicillin, and 50 µg/mL streptomycin sulfate. Hypoxic exposures (0.1% O₂, 5% CO₂, and balance N₂) were done in a Heto-Holten CellHouse 170 incubator (RS Biotech, Irvine, Scotland). Cell lines were obtained from the Cancer Research UK. Quinol compounds AJM290 and AW464 were provided by M.F.G. Stevens and proteasome inhibitor Z-Leu-Leu-Leu-Ala (MG132) was from Sigma (Gillingham, United Kingdom).

**Viability assay.** Cells were seeded at 2.5 × 10³ to 10 × 10³ per well 100 µL for 16-hour survival assay and 2.5 × 10³ per well 100 µL for 48-hour survival assay in 96-well plates 24 hours before experimental treatments. Cells were treated with compounds at 0.01 to 250 µmol/L in triplicates and further incubated in hypoxia or normoxia for 16 or 48 hours. Cell viability was measured by measuring metabolic conversion (by viable cells) of the dye MTS Cell Titer 96 AQueous One Solution Cell Proliferation Assay (Promega, Southampton, United Kingdom). In each well of a 96-well plate, 20 µL MTS was added, and plates were incubated for 2 to 4 hours in a cell culture incubator. MTS assay results were read in a 96-well format plate reader by measuring absorbance at 490 nm.

**Vascular endothelial growth factor ELISA.** Vascular endothelial growth factor (VEGF) secretion into the culture medium was measured.
using DuoSet ELISA Development Human VEGF Immunoassay (R&D Systems, Minneapolis, MN) and 3,3',5,5'-Tetramethylbenzidine Liquid Substrate System for ELISA (Sigma) by following the manufacturers' protocol. VEGF ELISA assay results were read in a 96-well format plate reader by measuring absorbance at 450 nm with correction at 540 nm.

**HRE reporter assay.** Cells were transfected with 2 µg/mL HIF-1 reporter plasmid or pGL3 promoter control plasmid and 0.02 µg/mL phRL-cytomegalovirus (CMV) Renilla luciferase plasmid using Fugene 6 eukaryotic transfection reagent kit (Roche, Welwyn Garden City, United Kingdom). The pGL3 *firefly* luciferase HIF-1 reporter plasmids contained the HRE from phosphoglycerate kinase or carbonic anhydrase IX (CA-IX). The pGL3 SV40 promoter vector was used for control and phRL-CMV Renilla luciferase plasmid was used as control for transfection efficiency (Promega). Twenty-four hours later, cells were exposed to hypoxia for 16 hours as described previously with AJM290 or AW464. Firefly and Renilla luciferase activity was measured using the Dual-Luciferase Reporter Assay System (Promega) according to the manufacturer’s instructions.

**Western blotting.** Whole-cell extracts were made by homogenizing cells in lysis buffer (6.2 mol/L urea, 10% glycerol, 5 mmol/L DTT, 1% SDS + protease inhibitors). Whole-cell extract was separated by 8% or 10% SDS-PAGE and transferred to polyvinylidene difluoride membrane. Primary antibodies used were mouse anti-HIF-1α, rabbit anti-HIF-2α (BD Transduction Laboratories, Lexington, KY), rabbit anti-hydroxylated-HIF-1α (provided by The Wellcome Trust Centre for Human Genetics, Oxford, United Kingdom), mouse anti-CA-IX M75 monoclonal antibody, mouse anti-BNIP3 (Sigma), mouse anti-Hsp70 (Abcam, Cambridge, United Kingdom), mouse anti-Hsp90 (Stressgen, San Diego, CA), and mouse anti-β-tubulin monoclonal antibody (Sigma). Immunoreactivity was visualized with horseradish peroxidase–linked goat anti-mouse or anti-rabbit serum and chemiluminescence.

**RNA extraction and reverse transcription.** Cells were rinsed with PBS and drained thoroughly. RNA was extracted from the cells using the solution D method described by Chomczynski and Sacchi (37). The quantity and quality of RNA extracted were assessed using NanoDrop.
ND 1000 Spectrophotometer (NanoDrop Technologies, Wilmington, DE) and the Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA), respectively. RNA samples were stored at –80°C. cDNA was synthesized by reverse transcribing RNA using the High-Capacity cDNA Archive kit (Applied Biosystems, Foster City, CA) following the manufacturer’s instruction.

Real-time quantitative PCR. Real-time quantitative PCRs were done in triplicate using the Corbett Research Rotor Gene RG-3000 (Sydney, New South Wales, Australia). Each reaction was done in an individual tube and made up to 25 μL containing 10 μL cDNA, 12.5 μL TaqMan PCR Master Mix (Abgene, Epsom, United Kingdom), 0.25 μL probe, 1 μL forward and reverse primer, and 0.2 μL H₂O. Conditions for the PCR were 2 minutes at 50°C, 10 minutes at 95°C, and 40 cycles, each consisting of 15 seconds at 95°C and 1 minute at 60°C. β-Actin was used as reference gene using primers (Invitrogen, Paisley, United Kingdom) forward 5′-CGATCCACCGAGTACTTG-3′ and reverse 5′-GCATCCACGGTACAG-3′ and reverse 5′-TGGAGTCGCGGATGATC-3′ with probe 63 (Exiqon, Vedbaek, Denmark). Primers against CA-IX were forward 5′-CTTGGAGGAATCTCAGGGAG-3′ and reverse 5′-GAGAAGACCCGGGATGATC-3′ with probe 22, VEGF forward 5′-CTACCTCACCAGGTACAG-3′ and reverse 5′-CCACCCCTCTCTATTTGCTG-3′ and reverse 5′-TGGAAGTAGCGGCTGAAGTC-3′ was amplified by PCR using Fuguene 6 eukaryotetransfectionreagentkit(Roche)withactivator 3.2-3T7 (2) was used as template for HIF-1α. The pGal plasmid contains sequences coding for the herpes simplex virus protein 16 amino acids 410 to 490 (VP16) as positive control was produced by insertion of this PCR product directly into pGal, preserving the reading frame, whereas pGal plasmid alone was used as background control. The pRL-CMV Renilla luciferase plasmid was used for transfection efficiency control (Promega). For transactivation assays, in 24-well plates, cells were transfected using Fugene 6 eukaryote transfection reagent kit (Roche) with activator plasmid (ranging between 0.5 and 5 ng), reporter plasmid (100-200 ng), and the transfection control plasmid pRL-CMV (0.5 ng). Twenty-four hours later, cells were exposed to 1 μmol/L. AJM290 in normoxia or hypoxia for 16 hours. Firefly and Renilla luciferase activity was measured using the Dual-Luciferase Reporter Assay System according to the manufacturer’s instructions.

Results

Effects of AJM290 and AW464 on viability and VEGF expression. Sixteen-hour exposure of MDA-MB-468 breast cancer cells seeded at 10 × 10³ to AJM290 or AW464 induced a dose-dependent decrease in viability and hypoxia-induced VEGF protein levels in cell medium (Fig. 1B and C). Expression of hypoxic VEGF was down-regulated at subtoxic concentrations by both compounds. In hypoxic cells treated with AJM290, VEGF IC₅₀ was 1.26 μmol/L and cell viability IC₅₀ was 4.1 μmol/L. Fifty percent inhibition of hypoxic VEGF levels to normoxic VEGF levels in cells treated with AJM290 was achieved at 0.6 μmol/L. AW464 was less toxic and a less potent inhibitor of VEGF than AJM290, with a VEGF IC₅₀ of 2.3 μmol/L and cell viability IC₅₀ of 8.2 μmol/L in hypoxic cells. Fifty percent inhibition of hypoxic VEGF levels to normoxic VEGF levels in cells treated with AW464 was achieved at 1.8 μmol/L. Cell viability and VEGF ELISA assays showed that both AJM290 and AW464 also inhibited hypoxic VEGF expression in the breast cancer cell line MDA-MB-231 and melanoma MDA-MB-435 cell line at subtoxic concentrations after 16 hours of exposure (Fig. 2A). AJM290 and AW464 also decreased VEGF at subtoxic doses in pVHL mutant RCC4 and 786-0 renal cell carcinoma cells that constitutively overexpress HIF-1α and/or HIF-2α protein, indicating that HIF-α is inhibited by AJM290 and AW464 independently of the pVHL pathway. Because 786-0 cell line expresses HIF-2α only and has been shown previously by us to regulate VEGF via HIF-2α (40), AJM290 and AW464 may also inhibit HIF-2α transcription activity. As Trx-1 and Trx-1 reductase have been shown to be up-regulated under hypoxic conditions (28), we investigated if hypoxic or pVHL mutant cells seeded at 2.5 × 10⁵ per well were more sensitive to the antiproliferative effect of AJM290 and AW464 following 16 or 48 hours of exposure to hypoxia or normoxia with compounds (Fig. 2B and C). The pVHL mutant renal cancer cell lines were only slightly more sensitive than the
pVHL-transfected cells to antiproliferative effects of AJM290 and AW464 following 16 hours of exposure. However, there were no significant difference between normoxic and hypoxic or pVHL-expressing and pVHL mutant cells following 16 or 48 hours of exposure.

**Effects of AJM290 and AW464 on HIF-1α, HIF-2α, and HIF-α downstream targets.** We incubated MDA-MB-468 cells under normoxic or hypoxic conditions for 16 hours in the presence or absence of 1 to 10 μmol/L AJM290 or AW464 and investigated the effects of these compounds on HIF-1α and HIF-2α protein levels and downstream transcription targets of HIF-α (Fig. 3A). Both HIF-1α and HIF-2α were undetectable under normoxia but were stabilized under hypoxia. Following 16 hours of treatment, surprisingly, both AJM290 and AW464 up-regulated the expression of HIF-1α and HIF-2α in both normoxic and hypoxic cells at a concentration of 2.5 to 10 and 10 μmol/L, respectively. However, the concentration at which this occurred varied between cell lines. No change in HIF-1β expression was observed following treatment with AJM290 (data not shown).

To investigate if both AJM290 and AW464 can inhibit HIF-α transcriptional activity, expression of downstream transcription targets of HIF-1α, CA-IX and BNIP3, was measured (Fig. 3A). Both AJM290 and AW464 inhibited the hypoxic up-regulation of CA-IX and BNIP3 in a dose-dependent manner. Compound AJM290 was shown to be a more potent inhibitor of HIF-α transcription activity than AW464 in MDA-MB-468 cell line, as both CA-IX and BNIP3 hypoxic up-regulation was inhibited by 1 μmol/L AJM290, whereas 10 μmol/L AW464 was required to inhibit the expression of CA-IX and BNIP3 in hypoxic cells. It is notable that these effects occurred at concentrations similar to those associated with an increase in HIF-α protein.

**Effects of AJM290 and AW464 compared with the proteasome inhibitor MG132.** To further understand the mechanism of HIF-α up-regulation in normoxic and hypoxic MDA-MB-468 cells following treatment with AJM290, we investigated the effects of AJM290 on heat shock proteins and hydroxylation of HIF-1α by PHDs and used proteasome inhibitor MG132 as a control (Fig. 3B). Both AJM290 and MG132 increased the expression of HIF-1α and hydroxylated-HIF-α in normoxic cells. Up-regulation of HIF-1α by MG132 and AJM290 coincided with the up-regulation of Hsp70, whereas no change in expression was observed with Hsp90. Maximum expression of Hsp70 in normoxic cells was observed following treatment with 2.5 μmol/L, whereas maximum expression of HIF-1α occurred following treatment with 10 μmol/L, although both proteins were induced at both drug concentrations. In hypoxic cells, expression of HIF-1α and Hsp70 was up-regulated by treatment with 2.5 μmol/L with no further enhancement by 10 μmol/L. Hsp70 was also up-regulated in MDA-MB-468 cells following treatment with AW464 (data not shown).

**Effects of AJM290 and AW464 on pVHL mutant cell lines.** To investigate how these compounds up-regulated HIF-α in cells that have defects in HIF degradation, we treated mutant pVHL RCC4+EV cells with 1 to 10 μmol/L AJM290 or AW464 for 16 hours (Fig. 3C). HIF-1α and HIF-2α expression is constitutively expressed in RCC4+EV and, like MDA-MB-468,
was up-regulated by AJM290 and AW464 but at a lower concentration of these drugs. In RCC4-EV cells, HIF-2α was strongly up-regulated following treatment with 1 μmol/L AJM290 and AW464 and weakly up-regulated with 2.5 μmol/L, whereas HIF-1α was up-regulated to maximum levels by 1 and 2.5 μmol/L AJM290 and 1 μmol/L AW464. The highest concentration of 10 μmol/L of either drug down-regulated the expression of HIF-1α and HIF-2α.

Unlike MDA-MB-468 cells, with 1 μmol/L AJM290, the increased HIF expression following treatment did not correlate with down-regulation of CA-IX. However, CA-IX expression was down-regulated at higher concentrations. AJM290 at 2.5 μmol/L did weakly down-regulate CA-IX, where HIF-1α is at its maximum level, whereas 10 μmol/L AJM290 and AW464 significantly down-regulated the expression of CA-IX, which coincided with the loss of HIF-1α and HIF-2α expression.

To investigate if both AJM290 and AW464 can also inhibit HIF-α transcription in hypoxic RCC4 that have been stably transfected with wild-type pVHL, cells were treated for 16 hours with 1 to 10 μmol/L AJM290 or AW464 in normoxic or hypoxic conditions (Fig. 3D). As in the RCC4-EV cells, both AJM290 and AW464 up-regulated HIF-1α and HIF-2α expression but at higher concentrations in normoxic cells, with maximum expression of HIF-α being observed following treatment with 2.5 μmol/L AJM290 or AW464. However, in hypoxic RCC4+pVHL cells, maximum HIF-α expression was observed following treatment with 1 μmol/L AJM290 or AW464. Similar to what was observed in MDA-MB-468 cells, lower concentrations of AJM290 and AW464 up-regulated HIF-α in hypoxic conditions. As with RCC4-EV cells, 10 μmol/L AJM290 and AW464 resulted in the loss of HIF-1α expression. However, in this pVHL-transfected cell line, the further up-regulation of HIF-α in hypoxia was associated with decreased transcriptional effect on downstream gene BNIP3 at 1 to 2.5 μmol/L. Expression of CA-IX could not be detected in RCC4 cells expressing pVHL. Hsp70 was also up-regulated in RCC4 cells following treatment with AJM290 or AW464 (data not shown).

Thus, in MDA-MB-468 cells and in the renal cancer cell line RCC4, reconstituted with wild-type pVHL, similar HIF-α responses occurred. However, in the RCC4-EV cell lines after HIF-α protein increased at the lower drug concentrations, at 10 μmol/L drug concentrations, HIF-α protein decreased, which was not observed in MDA-MB-468 cells. These results show that there are cell line differences following treatment with high concentrations of Trx-1 inhibitors and this may reflect differences in uptake and additional mechanisms involved in toxicity at higher doses.

**Impact of AJM290 and AW464 on mRNA of HIF-1α and HIF-1α target genes.** To test whether these compounds inhibited the activity of HIF-1α, in spite of up-regulating the protein, we incubated MDA-MB-468 cells under normoxic or hypoxic conditions for 16 hours in the presence or absence of 1 to 10 μmol/L AJM290 or AW464 and measured HIF-1α, BNIP3, CA-IX, and VEGF mRNA expression by real-time PCR (Fig. 3A-D). No significant increase in HIF-1α mRNA expression was observed in normoxic cells following treatment (Fig. 4A), which suggests that the increase in HIF-1α protein expression induced by AJM290 and AW464 in MDA-MB-468 cells was due to modulation of HIF-1α protein levels and not the HIF-1α mRNA. However, at the highest dose of the inhibitors, 10 μmol/L, a significant 44% reduction in HIF-1α RNA expression occurred.

We found that hypoxia significantly induced the expression of CA-IX, BNIP3, and VEGF mRNA in MDA-MB-468 cells relative to normoxic control (Fig. 4B and C). Both AJM290 and AW464 significantly inhibited hypoxic induction of CA-IX, BNIP3, and VEGF mRNA in a dose-dependent manner. Compound AJM290 was shown to be a more potent inhibitor of HIF-α transcription activity in MDA-MB-468 cell line.

**Inhibition of HIF-α transactivation.** HIF-α transactivation activity was measured in MDA-MB-468 cells by transiently transfecting a reporter construct expressing *firefly* luciferase under the control of multiple copies of HRE from phosphoglycerate kinase and CA-IX (Fig. 5A and B). Inhibitors had no effect on luciferase activity of CMV-driven *Renilla* phRL loading control or SV40 pGL3 promoter control (Fig. 5C). Both AJM290 and AW464 down-regulated phosphoglycerate kinase and CA-IX *firefly* luciferase in a dose-dependent manner. AJM290 and AW464 significantly inhibited hypoxia-induced HIF-α transactivation activity of phosphoglycerate kinase luciferase with IC₅₀ of 0.22 and 0.28 μmol/L, respectively.
and CA-IX luciferase with IC$_{50}$ of 0.44 and 0.85 µmol/L, respectively.

**Inhibition of HIF-HRE binding.** To further investigate the mechanism by which AJM290 inhibited HIF-1α transactivation, cytoplasmic and nuclear extracts from hypoxic MDA-MB-468 cells were treated with AJM290 and AW464. Extracts were first tested for expression of HIF-1α (Fig. 6A) before HRE-binding assays were carried out. Neither AJM290 nor AW464 reduced the expression of HIF-1α in the nucleus of hypoxic cells. However, expression of HIF-1α in the cytoplasm increased following treatment with AJM290 and AW464, which is consistent with increased HIF-1α expression in previous Western blots using whole-cell extracts. These results suggested at least a proportion of HIF-1α would be nonfunctional.

Because HIF-1α is expressed in the nucleus of hypoxic cells treated with AJM290 and AW464, we examined if HIF-1α in AJM290- and AW464-treated cells can bind to an oligonucleotide encompassing the HRE, HIF-1α-binding site (Fig. 6B). Using BD Mercury TransFactor kit specific for HIF-1α, HIF-1α in nuclear extracts from Cos-7 cells treated with CoCl$_2$ and hypoxic MDA-MB-468 cells were used as positive controls and were shown to bind to HRE, whereas normoxic MDA-MB-468 nuclear extract, which expressed low levels of HIF-1α, was used as negative control. The competitor oligonucleotides significantly inhibited HIF-1α binding of Cos-7 and MDA-MB-468 extracts. HIF-1α binding to HRE in nuclear extracts from MDA-MB-468 cells treated with AJM290 was inhibited in a dose-dependent manner, with significant down-regulation of HRE binding when cells were treated with AJM290 at 2.5 and 10 µmol/L. AW464 at 10 µmol/L only weakly but significantly inhibited HIF-1α binding to HRE. Direct addition of 10 µmol/L AJM290 and AW464 to nuclear extracts from HIF-1α-expressing pVHL mutant RCC4 cells or HIF-1α from hypoxic MDA-MB-468 did not affect the HIF-1α binding (data not shown), suggesting that AJM290 and AW464 do not directly interfere with the formation of protein-DNA complex.

**Effect of AJM290 on recombinant HIF-1α luciferase activity.** To further explore how AJM290 inhibit HIF-1α transactivation, MDA-MB-468 cells were transfected with plasmids expressing a fusion protein consisting of Gal linked to HIF-1α sequences and activity was tested by cotransfection with Gal4-responsive reporter gene (pUAS-tk-Luc; Fig. 7A). The pGal HIF-1α amino acids 28 to 826 activity was inducible by hypoxia and significantly repressed by 1 µmol/L AJM290 (Fig. 7B). We next tested to see if AJM290 could repress the COOH-terminal transactivation domain (CAD) of HIF-1α amino acids 775 to 826 linked...
to pGal (Fig. 7C). Hypoxia up-regulated pGal HIF-1α CAD activity and was repressed significantly by 1 μmol/L AJM290. Hypoxia and 1 μmol/L AJM290 had no significant effect on control pGal VP16 (Fig. 7D). This showed that the compounds can reduce pGal-HIF-1α CAD transactivation of Gal responsive sequences.

**Discussion**

Here, we investigated the effects of novel Trx-1 inhibitors AJM290 and AW464 on HIF-α expression and HIF-α transactivation in vitro. AW464 is a benzothiazole-substituted quinol compound that has been shown by mass spectrometry to bind to Trx-1 and has been proposed to cross-link irreversibly to cysteine residues 32 and 35 of Trx-1 active site via its two β-carbon atoms; the first link is reversible, whereas the second cross-link is thought to be irreversible (36, 41). AW464 can inhibit VEGF expression in hypoxic colorectal cells, and hypoxia has been shown to sensitize colorectal cancer cells to antiproliferative effect of AW464 (35). Further chemical syntheses and structure activity screening uncovered AJM290, an indole-substituted quinol that possess enhanced potency in vitro against human derived colon, renal, and mammary carcinoma cell lines and in vivo antitumor activity against breast, colon, and renal mouse xenografts (36, 42).

In this study, we showed that AJM290 and AW464 are potent inhibitors of the hypoxia pathway. Both compounds inhibited VEGF expression at subtoxic concentrations in hypoxic breast and melanoma cell lines and pVHL mutant renal cell lines, with AJM290 being the more potent compound. Studies carried out by Mukherjee et al. showed that hypoxia reduced the IC50 of AW464 by ~5-fold in all colorectal cancer cell lines tested (35). However, we saw no changes in IC50 between hypoxic and normoxic cell lines and pVHL mutant and stable pVHL-transfected cells following 16 or 48 hours of treatment with AJM290 or AW464. The difference in results could be due to the cell lines used (35).

AJM290 and AW464 in the MDA-MB-468 cell line inhibited expression of downstream HIF-α target genes, including the angiogenesis factor VEGF, pH regulatory protein CA-IX, and proapoptotic protein BNIP3, which coincided with the increase in HIF-α expression. Differences were observed between RCC4+EV and MDA-MB-468 cell lines. In RCC4+EV cells, lower concentration of AJM290 and AW464 increased HIF-1α, whereas CA-IX did not decrease. Higher concentrations of AJM290 did reduce CA-IX expression, whereas HIF-1α was at its highest level, but the highest concentrations of AJM290 and AW464 markedly reduced HIF-α expression, which coincided with the loss of CA-IX.

Thus, in MDA-MB-468 cells, the increase in HIF-1α expression resulted in the decrease of HIF-α transcription, whereas in RCC4+EV cell line it seems that it is the loss of HIF-α that leads to the decrease in HIF-α transcription targets. However, pVHL-transfected RCC4 cells behaved the same as MDA-MB-468 when treated with AJM290 and AW464 at lower concentrations. In hypoxic RCC4+pVHL cells, AJM290 and AW464 down-regulated BNIP3, whereas levels of HIF-α were up-regulated. The
differences in cell lines could be due to differences in drug uptake between MDA-MB-468 and RCC4 cells, with greater toxicity in the latter for the same concentration or differences in target availability. An effect of the pVHL mutation on the down-regulatory activity on HIF expression of higher concentrations of the inhibitors is unlikely, because the RCC4 cells showed the same effects of down-regulation of HIF-1α regardless of pVHL status. The lower concentrations did however reduce VEGF production, and because of the long half-life of CA-IX (3 days), down-regulation would be difficult to show from a high basal level.

Previous studies using Trx-1 inhibitor PX-12 and Trx-1 reductase inhibitor pleurotin have shown that these inhibitors, in addition to down-regulating HIF-1α transactivation, can also down-regulate HIF-1α protein level through an unknown mechanism (31). Presumably, the decreased transactivation was due to down-regulated protein. Thus, the effects we saw at high concentrations in RCC4 are similar to the previous reports and was the effect we expected, but considering the previous data on the role of Trx-1 in HIF stabilization, we were surprised to see both compounds here up-regulated HIF-α. The difference could be due to the different cysteine residues in Trx-1 that the compounds bind. Trx-1 inhibitor PX-12 irreversibly thioalkylates cysteine residue 73 of Trx-1 (43), whereas AW464 is postulated to target cysteine residues 32 and 35 (41); recently, it has been shown with mass spectrometry that up to 5 molecules of AW464 can covalently bind to Trx-1 (36).

Previous studies have shown that Trx-1 can affect HIF-1α transactivation acting through REF-1/HAP-1, which is a dual-functional protein harboring DNA repair endonuclease and cysteine-reducing activities (44–46). Trx-1 directly reduces REF-1 (47) and promotes the binding of transcription coactivator complex CBP/p300 to the CAD of HIF-1α leading to increased HIF-1α transactivation (6, 44, 45). Amino acid replacement cysteine in CAD of HIF-1α revealed that cysteine is essential for HIF-1α interaction with CBP (6). We showed that this specific domain, pGal HIF-1α amino acids 775 to 826, has increased transactivation activity when coupled to a pGal DNA-binding domain in hypoxic conditions, and AJM290 inhibits its transactivation activity. Inhibition of transactivation could be due to direct block in pGal DNA binding (although there was no effect on a control construct with this binding domain) or recruitment of transcription coactivators, which would result in the loss of pGal-HIF-1α-driven luciferase expression. In addition, we show that both AJM290 and AW464 inhibit HIF-1α binding to DNA in nuclear extracts from MDA-MB-468 hypoxic cells treated with these compounds. Direct addition of AJM290 and AW464 to nuclear extracts containing constitutive expression of HIF-1α had no effect in blocking HIF-1α HRE-DNA binding, suggesting that AJM290 and AW464 do not directly interfere with the formation of protein-DNA complex but may inhibit the formation of HIF-1α and a functional DNA-binding transcription complex.

The increase in HIF-1α protein following treatment with AJM290 and AW464 was not due to an effect on RNA level, which did not increase in normoxic MDA-MB-468 cells following treatment. High concentrations of AJM290 and AW464 did decrease HIF-1α RNA levels, which may explain what was observed in RCC4 cell lines following treatment with high concentration of these compounds. Neither AJM290 nor AW464 blocked PHDs from hydroxylating HIF-1α, which would have resulted in HIF-1α stabilization, as HIF-1α was shown to be hydroxylated when normoxic cells were treated.
with AJM290 and AW464. The increase in HIF-α protein expression could be due to a block in HIF-α protein degradation. However, it is unlikely to involve pVHL directly because AJM290 and AW464 increased HIF-α in RCC4 cell lines, which lack pVHL. Previous studies have shown that Hsp90/Hsp70 can stabilize HIF-1α by preventing its degradation via a pVHL-independent pathway (48). It is possible that Hsp70, which was shown to be up-regulated in cells treated with AJM290 or AW464, may prevent the degradation of HIF-α. Microarray analysis have shown that Hsp70 is induced by high doses of AW464 (35). Previous studies have also shown that HIF-1α can coimmunoprecipitate with Hsp90/Hsp70 and that Hsp70 directly binds to the oxygen-dependent degradation domain of HIF-1α (48), so it is possible that Hsp70 directly stabilizes HIF-α and inhibits degradation of HIF-α through a pVHL-independent pathway (49), but this would not account for the block in transactivation.

The phenotype induced by AJM290 and AW464 was similar to that produced by the proteasome inhibitor MG132. Like AJM290, MG132 up-regulated HIF-1α and Hsp70 in normoxic (Fig. 2B) and hypoxic cells and these changes were concordant with down-regulation of the target genes (data not shown). Previous studies have shown that MG132 blocks the proteasome degradation of hydroxylated HIF-α protein, resulting in increased levels of stabilized polyubiquitinated forms of HIF-1α, which have reduced transcriptional activity (50). HIF-1α up-regulated in hypoxic cells by AJM290 or MG132 (data not shown) was much less hydroxylated and therefore would be much less ubiquitinated by the ubiquitin/proteasome complex. Nevertheless, prolyl hydroxylases have some function even at low oxygen tension, which would then contribute to accumulation of modified ubiquitinated HIF. This residual function may explain the differences between pVHL mutant cells and the MDA-MB- 468 cells at lower drug concentrations. In the former, there is minimal ubiquitination, so proteasome inhibition, although up-regulating HIF, does not result in loss of function, whereas in MDA-MB-468 cells the accumulated HIF will be ubiquitinated. At higher drug concentrations, HIF was down-regulated as reported for other proteasome inhibitors.

Additionally, HIF-1α may not be in a reduced state following treatment with anti-Trx compounds, which is essential for its activity. Trx has also been shown to have a role in the proteasome function (51), and it is possible that inhibitors of Trx that bind to different cysteines will differentially modify proteasome interaction with key targets. This is the most likely explanation of an increase in hydroxylated HIF-α in normoxic cells and nonhydroxylated HIF-1α in hypoxic cells. The combined effects would then be a protein that is nonfunctional in DNA binding or transactivation but increased in amount. At higher concentration, additional effects occur with down-regulation of HIF-1α in RCC4 versus breast cancer cell lines.

We conclude that AJM290 and AW464 are effective drugs against the HIF pathway in breast cancer cells, and this is probably because of their unique activity compared with other Trx inhibitors. They decreased HIF-1α transactivation via the CAD, inhibited binding to HREs in DNA, increased cytoplasmic accumulation of HIF-α, and blocked the induction of HIF-α target genes. Both compounds have also been shown previously to have antitumor activity in vivo, and AW464 has been shown to exert antiproliferative effects on endothelial cells, whereas normal fibroblasts are resistant (33–36). Inhibition of Trx-1 redox system may therefore represent an effective way of treating cancer cells resulting in decreased hypoxia response, angiogenesis, and resistance to apoptosis.

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References


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