Identification of novel small-molecule inhibitors of hypoxia-inducible factor-1 transactivation and DNA binding

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Abstract

Hypoxia-inducible factor-α (Hif-α) plays an important role in tumor growth by increasing resistance to apoptosis and the production of angiogenic factors, such as vascular endothelial growth factor (VEGF). Therefore, Hif-α is an attractive target for development of novel cancer therapeutics. We have generated Chinese hamster ovary cells, which stably express luciferase reporter construct under the control of a hypoxia response element to screen 15,000 compounds. We identified 40 compounds that inhibited hypoxic up-regulation of luciferase, and the top 30 compounds were further screened in a secondary assay using MDA-468 breast cancer cell line. Eight compounds were shown to inhibit VEGF expression in hypoxic cells at subtoxic concentrations. Three top putative Hif inhibitors, DJ12, DJ15, and DJ30, were chosen for further analysis. Transient transfection of cells with hypoxia-regulated luciferase reporter plasmids further validated that these compounds inhibit hypoxia up-regulated genes. All three compounds failed to inhibit Hif-1α protein levels but they did inhibit induction of downstream targets of Hif-α under hypoxia. Two of the three compounds were cell type specific, whereas compound DJ12 inhibited VEGF at subtoxic levels in breast cancer cell lines MDA-468 and ZR-75, melanoma cell line MDA-435, and pVHL mutant renal cancer cell lines RCC4 and 786-0. Compound DJ12 down-regulated mRNA of downstream targets of Hif-α, and significantly inhibited Hif-1α transactivation activity by blocking Hif-1α hypoxia response element-DNA binding. Our cell-based approach and deconvolution of the inhibitory effect of DJ12 has identified a novel compound that targets the hypoxia pathway by inhibiting Hif-α–inducible transcription. [Mol Cancer Ther 2006;5(9):2193–202]

Introduction

Hypoxia-inducible factor (Hif) is an α,β heterodimeric transcription factor that directs a broad range of responses in hypoxic cells (1–3). 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 (4). Hif-1β is a constitutive nuclear localized subunit, which binds to available Hif-1α (5). 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 Fe^{2+} and 2-OG-dependent dioxygenases (PHD1-3), which leads to Hif-α degradation via the pVHL (von Hippel-Lindau) E3 ubiquitin ligase complex and the 26S proteosome (6). Limiting oxygen levels or the availability of Fe^{2+} with iron chelators (7, 8) allows Hif-α to escape proteolysis. In the nucleus Hif-α, β heterodimer interacts with coactivators, such cAMP-responsive element binding protein–binding protein/p300, and becomes transcriptionally active (9). Upon 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. 10), 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 (11–15). Hif-1α 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 (11–16). Besides physiologic hypoxia, genetic abnormalities frequently detected in human cancers, which include key oncogenes (*HER2, FRAP, H-RAS*, and *SRC*) and tumor suppressor genes (*pVHL, p53*, and *PTEN*), are also associated with induction of Hif-1α activity and expression of Hif-1α–inducible genes (17–22).

Due to the involvement of Hif in tumor progression and angiogenesis, Hif is a promising molecular target for development of cancer therapeutics (23–25). Different approaches have been used to inhibit Hif activity. Disruption of Hif-1α transcriptional activity, using a peptide that interferes with the interaction between Hif-1α and the coactivator p300/cAMP-responsive element binding protein–binding protein, has shown therapeutic

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activity in xenograft models of colon carcinoma and breast cancer (26). Other approaches include pharmacologic inhibition of the signal transduction pathway that control Hif activity (phosphatidylinositol 3-kinase and mammalian target of rapamycin inhibitors), chaperone proteins (HSP90 inhibitors), redox state (thioredoxin and thioredoxin reductase inhibitors), and microtubule stability (2ME2; refs. 18, 27–32). One of the approaches to identify inhibitors of the Hif pathway has been to screen large libraries of small molecules using a high-throughput, cell-based assay (33–36). Such approach has led to the discovery of a quinocarmycin analogue and closely related camptothecin analogues that are topoisomerse inhibitors.

We screened 15,000 compounds using a cell-based, high-throughput method to identify small-molecule inhibitors of the hypoxia pathway. Chinese hamster ovary cells were genetically engineered to stably express a recombinant vector in which the luciferase reporter gene is under control of three copies of HRE. This was then followed with a validation screening of 30 top compounds using endogenous markers and transient transfections with HRE-driven luciferase in hypoxic breast, melanoma, and renal cancer cell lines. We identified one compound that inhibited the hypoxia pathway in several cell types and inhibited Hif-1α transactivation of downstream target genes.

Materials and Methods

Cell Culture

Human breast cancer cell lines MDA-MB-468 and ZR-75, and melanoma cell line MDA-MB-435, were maintained in DMEM with supplements. The pVHL-deficient RCC4 and 786-0 renal carcinoma cell lines and their counterpart containing a stably transfected *pVHL* gene were cultured in αMEM and DMEM (containing supplements), respectively, and maintained in selection with 500 μg/mL G418. DMEM and αMEM were supplemented with 10% fetal bovine serum, L-glutamine (2 mmol/L), penicillin (50 IU/mL), and streptomycin sulfate (50 μg/mL). Selection agents were omitted from the culture medium when cells were resuspended for experiments. Hypoxic exposures (0.1% O_2, 5% CO_2, and balance N_2) were done in a Galaxy R incubator (RS Biotech, Irvine, Scotland) or a Heto-Holten CellHouse 170 incubator (RS Biotech).

Viability Assay

Cells were seeded at 10×10^3 per well, 100 μL, 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 hours. Compounds cycloheximide and quinocarmycin analogue NSC-607097 (33) were used as controls. Cell viability was measured by measuring metabolic conversion (by viable cells) of the dye MTS [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2*H*-tetrazolium, inner salt] from Cell Titer 96 AQueous One Solution Cell Proliferation Assay kit (Promega, Southampton, United Kingdom). In each well of a 96-well plate, 20 μL of MTS was added, and plates were incubated for 2 to 4 hours in 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), by following the protocol of the manufacturer, and 3,3′,5,5′-tetramethylbenzidine Liquid Substrate System for ELISA (Sigma, Gillingham, United Kingdom). VEGF ELISA assay results were read in a 96-well format plate reader by measuring absorbance at 450 nm with correction at 540 nm.

Western Blotting

Cells were homogenized in lysis buffer (6.2 mol/L urea, 10% glycerol, 5 mmol/L DTT, and 1% SDS plus protease inhibitors). Whole cell extract was separated by 8% or 10% SDS-PAGE and transferred to polyvinylidene difluoride membrane. Primary antibodies were mouse anti-Hif-1α monoclonal antibody (BD Transduction Laboratories, Lexington, KY), mouse anti-CAIX M75 monoclonal antibody (a gift from Dr. J. Pastorek, Institute of Virology, Slovak Academy of Science, Bratislava, Slovak Republic; ref. 37), mouse anti-BNIP3 (Sigma), and mouse anti-β-tubulin monoclonal antibody (Sigma). Immunoreactivity was visualized with horseradish peroxidase–linked goat antimouse serum and chemiluminescence.

HRE Reporter Assay

Cells were transfected with 2 μg/mL Hif-1α reporter plasmid or pGL3 promoter control plasmid, and 0.02 μg/mL phRL-cytomegalovirus *Renilla* luciferase plasmid using Fugene 6 eukaryote transfection reagent kit (Roche, Welwyn Garden City, United Kingdom). The pGL3 *firefly* luciferase Hif-1α reporter plasmids contained the HRE from phosphoglycerate kinase (PGK) or CA-IX. The pGL3 promoter vector was used for control and phRL-cytomegalovirus *Renilla* luciferase plasmid was used as control for transfection efficiency (Promega). Twenty-four hours later, cells were exposed to compounds in hypoxic or normoxic conditions for 16 hours as described previously. Firefly and *Renilla* luciferase activity were measured using the Dual-Luciferase Reporter Assay System (Promega) according to instructions from the manufacturer.

RNA Extraction

Cells were rinsed with PBS and drained thoroughly. RNA was extracted from the cells using the solution D method described by Chomczynski and Sacchi (38). The quantity and the quality of RNA extracted was 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.

RNase Protection Assay

Total RNA was dissolved in hybridization buffer [80% formamide, 40 mmol/L PIPES, 400 mmol/L sodium chloride, and 1 mmol/L EDTA (pH 8)]. Fifteen micrograms of RNA were assayed by RNase protection using [^{32}P]CTP-labeled riboprobes for lactate dehydrogenase (LDH) and VEGF. To attenuate the signal strength of the highly

abundant loading control U6 small nRNA, a riboprobe of significantly lower specific activity was prepared by addition of unlabeled CTP to the labeling reaction. The protected fragment size for VEGF121 was 517 nucleotides, and for VEGF165 and VEGF189 was 439 nucleotides. The protected fragment for LDH was 285 and the U6 small nRNA was 106 nucleotides. After resolution on 8% polyacrylamide gels, bands were analyzed using a PhosphorImager (Molecular Dynamics, Sunnyvale, CA).

Reverse Transcription and Real-time Quantitative PCR

cDNA was synthesized by reverse transcribing RNA using the High Capacity cDNA Archive kit (Applied Biosystems, Foster City, CA) following instructions from the manufacturer. Real-time quantitative PCR (qPCR) reactions were done in triplicate using the Corbett Research Rotor Gene RG-3000 (Sydney, 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 H_2O. Conditions for the PCR reaction were 2 minutes at 50°C, 10 minutes at 95°C, and then 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) 5′-CCCAGCACAATGAAGATCAA-3′ forward and 5′-CGATCCACACGGAGTACTTG-3′ reverse with probe 63 (Exiqon, Vedbaek, Denmark). Primers against VEGF 5-CTACCTCCACCATGCCAAGT-3′ forward and 5′-CCACTTCGTGATGATTCTGC-3′ reverse with probe 29, and BNIP3 5′-TGCTGCTCTCTCATTTGCTG-3′ forward and 5′-GACTCCAGTTCTTCATCAAAAGGT-3′ reverse with probe 22 were used for qPCR. Relative quantitation of gene expression was done using the method described by Pfaffl (39). In brief, comparisons were made between the number of cycles required for the fluorescence of a sample to reach a predetermined threshold that lay within the exponential phase and above nonspecific background. The relative ratio of gene expression was calculated as follows:

$$\text{relative ratio} = \frac{(E_{\text{target}})^{D_{\text{Ct target}}(\text{mean comparator} - \text{mean sample})}}{(E_{\text{ref}})^{D_{\text{Ct ref}}(\text{mean comparator} - \text{mean sample})}}$$

where E_{target} is the reaction efficiency of the gene of interest, E_{ref} is the reaction efficiency of the reference gene, and D_{Ct} is the cycle difference between the comparator and the sample. All calculations are based on the mean value of PCR reactions done in triplicate.

Nuclear Cell Extracts and HRE Binding Assay

MDA-468 cells were grown to 80% confluence in T-75 flasks and treated with compounds for 16 hours in hypoxia. Nuclear extracts were prepared using a TransFactor Extraction kit (BD Biosciences Clontech, Palo Alto, CA) according to the instructions of the manufacturer. Cells were collected and lysed, and the cytosolic fraction was removed. The nuclear pellets were resuspended and homogenized, and the nuclear extracts were collected. Cytoplasmic and nuclear cell extracts from MDA-468 cells were run on 8% SDS-PAGE and transferred to polyvinylidene difluoride membrane and staining with anti-Hif-1α (BD Transduction Laboratories), anti-β-tubulin (Sigma), anti–lamin A+C (Abcam, Cambridge, United Kingdom), and anti–histone deacetylase-1 (Santa Cruz Biotechnology, Santa Cruz, CA) monoclonal antibodies.

Hif-1α DNA-binding activity was determined using a BD Mercury TransFactor kit specific for Hif-1α (BD Biosciences Clontech) according to the instructions from the manufacturer. Three 30-μg aliquots of nuclear extracts from each treatment group were added to individual wells of a 96-well plate coated with oligonucleotides containing the consensus-binding sequence of Hif-1α. Bound Hif-1α was detected by the appropriate primary and horseradish peroxidase–labeled secondary antibodies. 3,3′,5,5′-Tetramethylbenzidine substrate was added and converted by horseradish peroxidase to a blue product. After 30 minutes, the absorbance was measured at 650 nm. Nuclear extracts from Cos-7 cells treated with $CoCl_2$ (Active Motif, Carlsbad, CA) and nuclear extracts incubated with a Hif-1α–specific competitor oligonucleotide (500 ng) served as positive and negative controls, respectively.

Results

Secondary Screening—Effects of Putative Hif Inhibitors on VEGF Hypoxic Expression and Cell Growth of MDA-468 Cell Line

VEGF secretion under hypoxia as a marker of Hif function and MTS assay for viability was used to screen the top 30 compounds that came out of 15,000 from the Chinese hamster ovary HRE-Luciferase high-throughput primary screen. Eight of 30 putative Hif inhibitors were shown to inhibit VEGF expression in MDA-468 cell line at subtoxic levels following 16-hour exposure, where IC_{50} viability was 2-fold more than VEGF IC_{50}. Based on the inhibition of VEGF in hypoxic cells versus compound toxicity, three top compounds, DJ12, DJ15, and DJ30, were selected for further analysis and compared with quinocarmycin analogue NSC-607097 (structures of the compounds are shown in Fig. 1A; ref. 33). Sixteen-hour exposure of MDA-468 breast cancer cells to D12, DJ15, DJ3, or NSC-607097 induced a dose-dependent decrease in hypoxia-induced VEGF protein levels and viability (Fig. 1B–D). Compounds DJ12, DJ15, DJ30, and NSC-607097 inhibited hypoxia-driven VEGF secretion following compound exposure with IC_{50} values of 53, 40, 2.3, and 0.42 μmol/L, respectively, and concentration values of 21, 21, 0.9, and 0.27 μmol/L, respectively, for inhibiting of VEGF in hypoxic cells to 50% of normoxic levels (Fig. 1B). DJ30 was shown to be toxic with IC_{50} of 6 μmol/L in normoxic cells and 9.5 μmol/L in hypoxic cells, whereas DJ12 and DJ15 compounds were shown to be toxic only at high concentrations with IC_{50} of 191 and 150 μmol/L, respectively, in normoxic cells, and above 250 μmol/L in hypoxic cells (Fig. 1C). Control compound NSC-607097 was nontoxic following 16 hours of treatment but did have

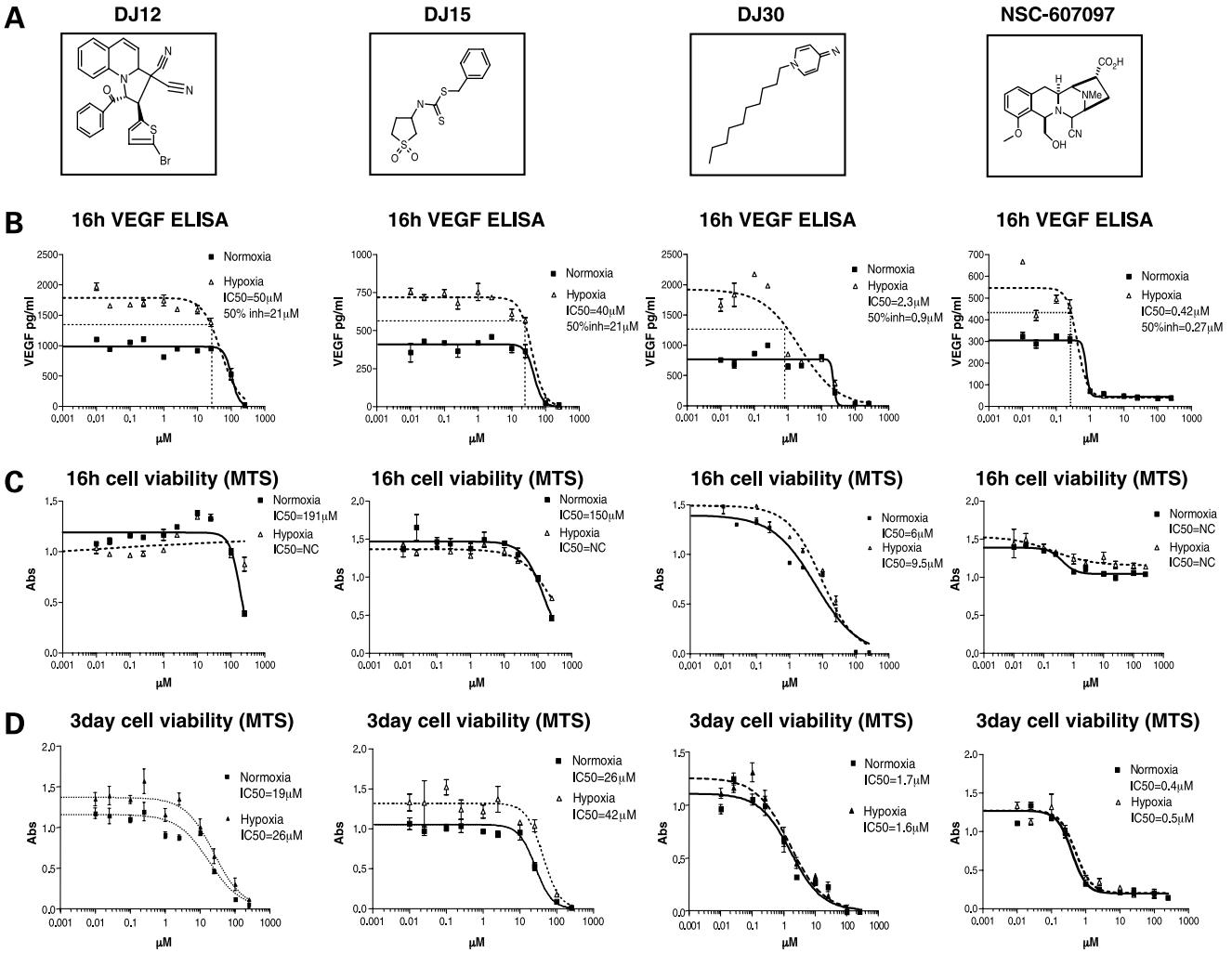

Figure 1. Chemical structures of DJ12, DJ15, DJ30, and NSC-607097 (**A**); and effect of DJ12, DJ15, DJ30, and NSC-607097 on MDA-468 VEGF expression (**B**), cell viability following 16 h of treatment in normoxic or hypoxic conditions (**C**), and cell viability following additional 56 h of incubation in drug-free medium (**D**). Results were plotted in triplicate. *Bars,* SE. Viability IC_{50}, VEGF IC_{50} and 50% VEGF hypoxic to normoxic values were calculated using Graph Prism.

an effect on cell proliferation from concentrations of >1 μmol/L. Control compound cycloheximide was also nontoxic following 16 hours of treatment but did reduce VEGF in hypoxic cells with VEGF IC_{50} of 0.19 μmol/L, and like NSC-607097 had an effect on cell proliferation above 1 μmol/L (data not shown).

However, upon 16-hour incubation with compound followed by additional 56-hour incubation with compound-free cell medium, all four compounds were shown to be toxic, with IC_{50} of 19 to 26 μmol/L for DJ12, 26 to 42 μmol/L with DJ15, 1.6 to 1.7 μmol/L with DJ30, and 0.4 to 0.5 μmol/L with NSC-607097 (Fig. 1D).

Inhibition of Hif-α Transactivation

Hif-α transactivation activity was measured in MDA-468 cells by transiently transfecting a reporter construct expressing *firefly* luciferase under the control of multiple copies of HRE from PGK (Fig. 2). Hypoxia had no effect on luciferase activity of cytomegalovirus-driven *Renilla* phRL loading control or pGL3 promoter control construct. All three compounds down-regulated PGK *firefly* luciferase in a dose-dependent manner relative to luciferase of pGL3 SV40 promoter control. DJ12, DJ15, and DJ30 significantly inhibited hypoxia-induced Hif-α transactivation activity of PGK luciferase with IC_{50} of 3.6, 22.5, and 1 μmol/L, respectively. All three compounds also down-regulated reporter construct expressing *firefly* luciferase under the control of multiple copies of HRE from CA-IX (data not shown).

Effects of Compounds on Hif-1α and Hif-α Downstream Targets

To explore the underlying mechanism of DJ12, DJ15, and DJ30 inhibition of hypoxic VEGF, we incubated MDA-468 cells under normoxic or hypoxic conditions for 16 h in the presence or absence of increasing concentration of

compounds and investigated the effects of these compounds on Hif-1α protein levels and downstream transcription targets of Hif-α (Fig. 3A–C). Hif-1α was shown to be undetectable under normoxia and stabilized under hypoxia. Following 16 hours of treatment, DJ12 and DJ30 had no effect on induction of Hif-1α expression, whereas DJ15 down-regulated Hif-α at a high concentration of 250 μmol/L in hypoxic cells. Control compound cycloheximide down-regulated Hif-α protein, whereas NSC-607097 did not.

To investigate if the compounds inhibited Hif-α transcription activity, expression of downstream transcription targets of Hif-1α, CA-IX, and BNIP3 were analyzed by Western blotting. All three compounds down-regulated CA-IX and BNIP3 in hypoxic cells in a dose-dependent manner. Compound DJ30 was shown to be a more potent inhibitor than DJ12 and DJ15, as both CA-IX and BNIP3 were down-regulated by 1 μmol/L DJ30, whereas 10 μmol/L DJ12 and DJ15 were required to reduce the expression of CA-IX and BNIP3 in hypoxic cells. Control compounds cycloheximide and NSC-607097 at 10 μmol/L down-regulated both CA-IX and BNIP3 in hypoxic cells.

Cell Line Comparison

To see if all three compounds inhibited hypoxic VEGF levels at subtoxic concentrations in different cancer cell lines, all three compounds were tested on breast cancer cell lines MDA-468 and ZR-75, MD435 melanoma cell line, and pVHL mutant renal cancer cell lines RCC4 and 786-0 that constitutively overexpress Hif-α proteins (Fig. 4A-D). Compound DJ12 inhibited VEGF in hypoxic and pVHL mutant cell lines at subtoxic concentration with VEGF IC_{50} ranging from 20 to 50 μmol/L, whereas viability IC_{50} ranged from 165 to above 250 μmol/L (Fig. 4A). However, compound DJ15 was shown to be toxic to renal cancer cell line RCC4 and did not inhibit VEGF at subtoxic concentrations (Fig. 4B). Compound DJ30 was only specific in inhibiting VEGF at subtoxic levels in PTEN-negative breast cancer cell lines MDA-468 and ZR-75 (Fig. 4C). Compound NSC-607097 inhibited VEGF in hypoxic and pVHL mutant cell lines with a VEGF IC_{50} range of 0.2 to 1 μmol/L with no toxic effects (Fig. 4D). However, NSC-607097 did have a mild antiproliferative effect on all cell lines at concentrations >1 μmol/L.

Effect of DJ12 on mRNA Expression of Hif-α Target Genes

Because DJ15 and DJ30 failed to inhibit hypoxic or pVHL mutant expression of VEGF at subtoxic levels in all cell types tested, only compound DJ12 was further investigated. To test whether DJ12 inhibited up-regulation of Hif-1α target genes at the mRNA level, we incubated MDA-468 cells under normoxic or hypoxic conditions for 16 hours in the presence or absence of 10 to 100 μmol/L DJ12 and measured VEGF and LDH mRNA expression by RNase protection assay and VEGF and BNIP3 mRNA expression by real-time quantitative PCR. The induction of both VEGF and LDH mRNA levels under hypoxic conditions, analyzed by RNase protection assay, were shown to be inhibited when cells were treated with 100 μmol/L DJ12 (Fig. 5A). Similar results were obtained using real-time PCR; induction of both VEGF and BNIP3 mRNA levels in hypoxic cells was inhibited when cells were treated with 100 μmol/L DJ12 (Fig. 5B).

Inhibition of Hif-HRE Binding

To further investigate the mechanism by which DJ12 inhibit Hif-1α transactivation, cytoplasmic and nuclear extract from hypoxic MDA-468 cells treated with DJ12 were first tested for expression of Hif-1α (Fig. 5C) before the HRE binding assays were carried out. Compound DJ12 at 100 μmol/L did not block the translocation or expression of Hif-1α in the nucleus of hypoxic cells. Treatment of cells with DJ12 also increased the expression of Hif-1α in the cytoplasm. Because Hif-1α is expressed in the nucleus of hypoxic cells treated with DJ12, we examined if Hif-1α in DJ12-treated cells can bind to an oligonucleotide encompassing the HRE of the Hif-1α binding site (Fig. 5D). Using BD Mercury TransFactor kit specific for Hif-1α, Hif-1α in

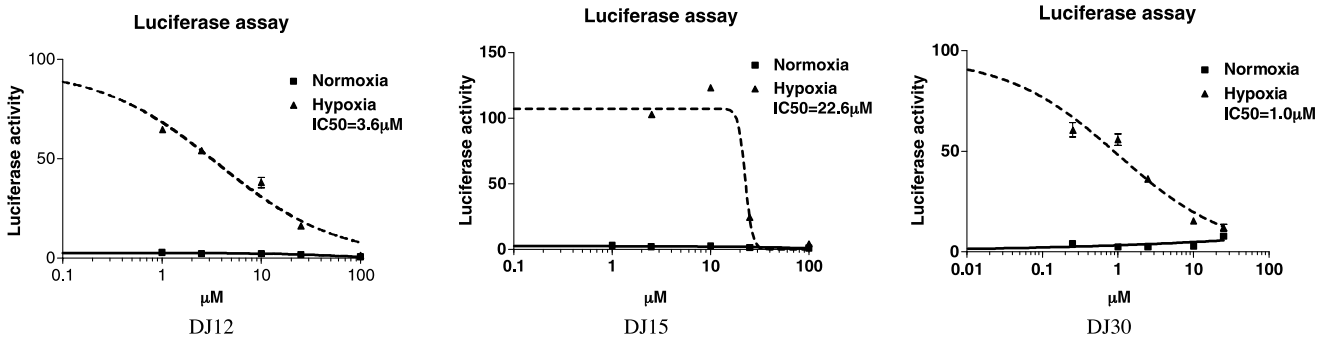

Figure 2. Effect of DJ12, DJ15, and DJ30 on transactivation of Hif-1α. MDA-468 cells were transiently transfected with vector-expressing *firefly* luciferase under the control of the HRE from PGK or SV40 as control and exposed to normoxia or hypoxia for 16 h in the presence of varying concentrations of compounds. The cells were cotransfected with a vector expressing *Renilla* luciferase. Transfection efficiency was normalized using *Renilla* luciferase, and the activation of the HRE was determined by calculating the increase in *firefly:Renilla* luciferase activity compared with the SV40 promoter vector control activity under same conditions (relative fluorescence). Duplicate data points from a single experiment. *Bars,* SE. IC_{50} values were calculated using Graph Prism.

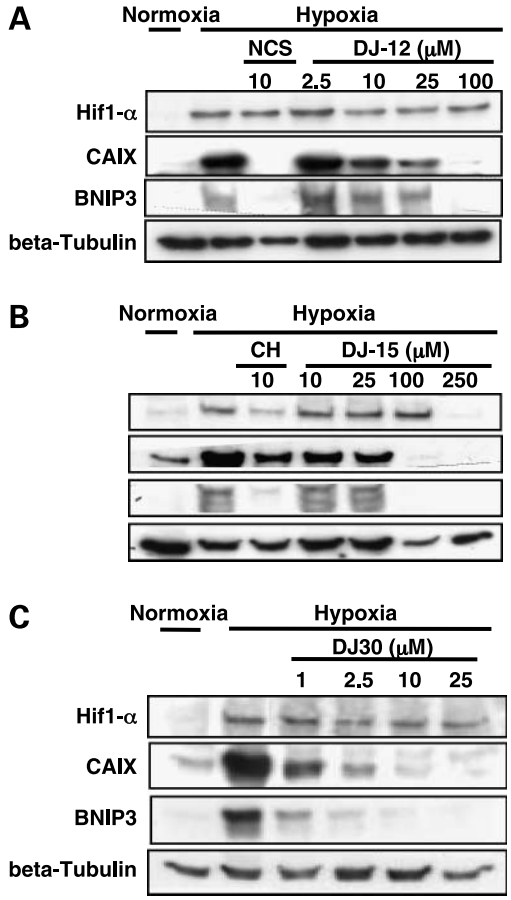

Figure 3. Effect of DJ12, DJ15, and DJ30 on Hif-1α and Hif-1α target genes *CA-IX* and *BNIP3* in MDA-468 cell line. Cells were exposed to normoxia or hypoxia for 16 h in the presence of increasing concentrations of DJ12 (**A**), DJ15 (**B**), or DJ30 (**C**). Compound NSC-607097 (*NSC*) and cycloheximide (*CH*) were used as positive control compounds. β-Tubulin was used as loading control for Western blots.

nuclear extracts from Cos-7 cells treated with $CoCl_2$ and hypoxic MDA-468 cells were used as positive controls and were shown to bind to HRE, whereas normoxic MDA-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-468 extracts by 97% and 94%, respectively. Hif-1α binding to HRE in nuclear extracts from MDA-468 cells treated with 100 μmol/L DJ12 was significantly inhibited by 45%. Direct addition of 100 μmol/L DJ12 to nuclear extracts from Hif-1α expressing pVHL mutant RCC4 cells did not affect the Hif-1α binding (data not shown), suggesting that DJ12 does not directly interfere with the formation of protein-DNA complex.

Discussion

The aim of this study was to identify a novel small-molecule inhibitor of the Hif-α transcriptome. From the high-throughput primary screen of 15,000 compounds, we

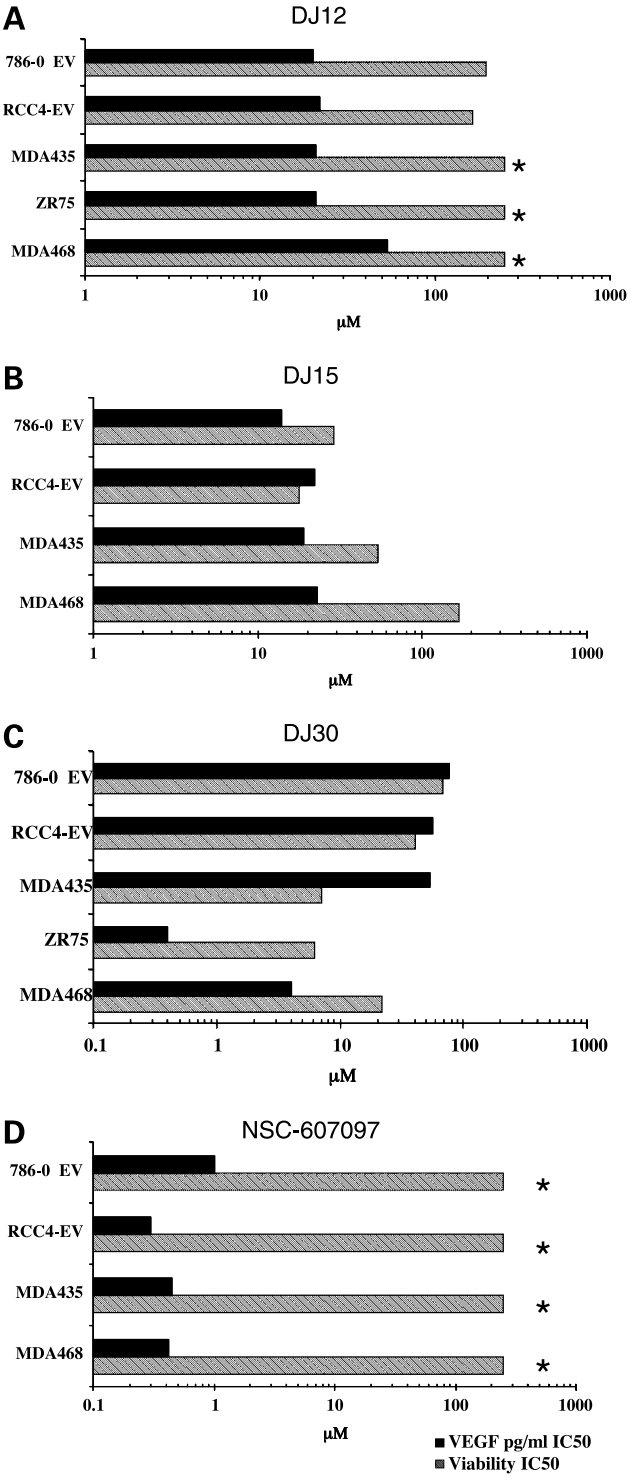

Figure 4. Effect of compound DJ12 (**A**), DJ15 (**B**), DJ30 (**C**), and NSC-607097 (**D**) on cell viability and VEGF IC_{50} of hypoxic breast cancer cell line MDA-468 and ZR-75, melanoma cancer cell line MDA-435, and pVHL mutant cell line RCC4 and 786-0 following 16 h of treatment. Viability and VEGF IC_{50} values were calculated by plotting the effect of increasing drug doses in triplicate on cell viability and VEGF expression using Graph Prism software. *, IC_{50} could not be calculated due to low toxic effects of highest dose (250 μmol/L) of compound.

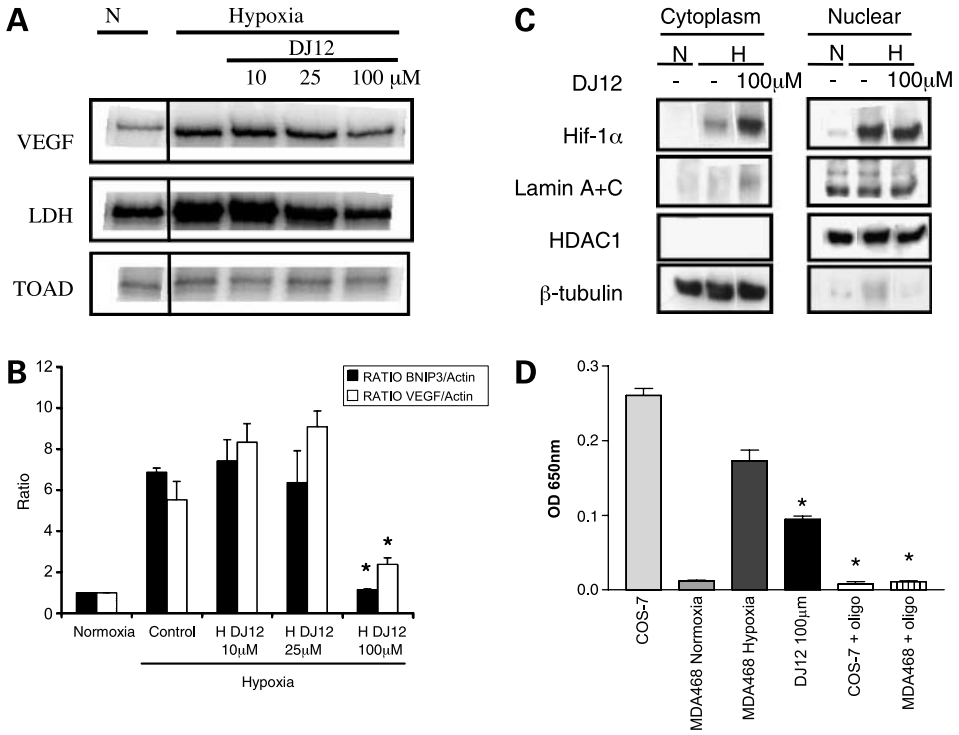

Figure 5. Effect of DJ12 on Hif-1α targets genes *VEGF, LDH*, and *BNIP* mRNA expression analyzed by RNase protection assay (**A**) and real-time PCR (**B**), and on Hif-1α nuclear localization (**C**) and Hif-1α DNA binding (**D**). Cells were exposed to normoxia or hypoxia for 16 h in the presence of DJ12. U6 snRNA internal loading control was used in RNase protection assay and β-actin was used as loading control in real-time PCR. *Columns,* mean of BNIP3/β-actin and Hif-1α/β-actin ratios from triplicate PCR reactions from a single experiment; *bars,* SE. *, significant difference from hypoxic control value using Student's *t* test ($P < 0.05$). Protein levels in cytoplasmic and nuclear cell extracts were detected using Western blotting with antibody against Hif-1α. β-Tubulin was used as loading control for cytoplasmic cell extracts, and lamin A + C and histone deacetylase-1 was used as loading control for nuclear cell extracts. Hif-1α DNA binding assay was carried out on the same MDA-468 nuclear extracts treated with DJ12 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-468 cells were used as positive controls. Nuclear extracts from normoxic MDA-468, and nuclear extracts incubated with a Hif-1α–specific competitor oligonucleotide served as negative controls. *Columns,* mean of triplicate set of data from a single experiment; *bars,* SE. *, significant difference from hypoxic or $CoCl_2$-treated control absorbance (*OD*) values using Student's *t* test ($P < 0.05$).

identified 30 compounds capable of inhibiting hypoxic HRE-driven luciferase expression in stable HRE-luciferase transfected Chinese hamster ovary cell lines. Looking at endogenous genes in MDA-468 cell line regulated by hypoxia, 8 of 30 small molecules down-regulated VEGF expression in hypoxic cells at subtoxic concentrations following 16-hour exposure. Three of the top compounds, DJ12, DJ15, and DJ30, were further investigated. All three compounds inhibit hypoxic VEGF, CA-IX, and BNIP3 expression, but not Hif-1α protein levels. All three compounds specifically inhibited PGK-luciferase in hypoxic cells and not the control cytomegalovirus-luciferase. Control compounds cycloheximide, a protein synthesis inhibitor, and NSC-607097 (33), a quinocarmycin analogue, also down-regulated Hif-α target genes by down-regulating Hif-1α protein expression and Hif-α transactivation activity, respectively, in the MDA-468 cell line.

Compounds DJ15 and DJ30 were also shown to be cell type specific. Compound DJ30 failed to down-regulate VEGF at subtoxic concentrations in RCC4 and 786-0 renal cancer cell lines and MD435 melanoma cell line, and compound DJ15 failed to down-regulate VEGF at subtoxic concentrations in RCC4 cells. It is possible that both these compounds target specific cell signaling pathways that control Hif-α transactivation activity in certain cell types. Compound DJ30 was shown to be a potent inhibitor of Hif transactivation in PTEN-negative breast cancer cell line MDA-468 and ZR-75 but not in PTEN-negative renal cancer cell line 786-0 (40). The phosphatidylinositol 3-kinase/Akt pathway is negatively regulated by PTEN and the AKT pathway induces the expression of Hif-α. The phosphatidylinositol 3-kinase inhibitor LY294002 down-regulates Hif-α protein expression via inhibiting translation of Hif-1α mRNA and Hif-α protein stability (18, 27, 41). However, DJ30 did not down-regulate Hif-α protein expression in hypoxic cells, although it did induce down-regulation of phosphorylated AKT at higher concentrations than that required to down-regulate downstream targets of Hif-α (data not shown). DJ30 may target other signaling pathways that modulate Hif-1α transactivation in MDA-468 and ZR-75 breast cancer cell line, but not AKT.

One mechanism by which compound DJ12 may inhibit Hif-1α transactivation is through blocking Hif-1α HRE-DNA binding. Here, we showed 45% inhibition of Hif-1α

DNA binding from nuclear extracts of hypoxic MDA-468 cells treated with 100 μmol/L DJ12. Direct addition of DJ12 to nuclear extracts containing constitutive expression of Hif-1α had no effect in blocking Hif-1α HRE-DNA binding, suggesting that DJ12 does not directly interfere with the formation of protein-DNA complex but may inhibit the formation of Hif-1α, Hif-1β, and CBP/p300 transcription complex or folding of Hif-1α.

Studies using high-throughput screens carried out by other groups have also identified Hif-1α inhibitors that have antiproliferative effects, and can inhibit Hif signaling pathway via different mechanisms (30, 33–35). Studies by Chau et al. (35) identified two novel compounds that inhibited Hif-1α transactivation and protein stability in cells treated with iron chelator deferoxamine mesylate following 16-hour exposure. However, one compound did not inhibit Hif-α activity or down-regulate Hif-1α protein in hypoxic cells. The inactivation of PHD enzymes, which results in the stabilization of Hif-1α, is a common feature of hypoxia and deferoxamine mesylate treatments. However, iron chelators can affect other cellular pathways, as labile iron is indispensable for DNA synthesis and a host of metabolic processes (42–44). Therefore, chelating the labile iron pool within cells may not be an ideal method of simulating hypoxic conditions. In addition, both compounds inhibited insulin-like growth factor-I–induced Hif-1α expression, which suggests that one compound regulates Hif-1α protein stability through a specific pathway, whereas the second compound is Hif-1α protein specific. However, long-term, 72-hour exposure to these compounds, including NSC-607097, was shown to be toxic and inhibit cell growth through an unknown mechanism (35). The compounds discovered by Rapsidara et al. (33) included camptothecin analogues that inhibit topoisomerase I activity. Inhibition of topoisomerase I activity by the camptothecin analogues resulted in suppression of Hif-α activity and Hif-1α protein expression in hypoxic cells; and inhibition of Hif-1α, angiogenesis, and tumor growth in xenografts (45). This mechanism is through a novel pathway connecting topoisomerase I–dependent signaling events and the regulation of Hif-1α protein expression and function, which is independent of replication-mediated DNA damage (46). Studies by Tan et al. (34) also identified a compound that strongly reduced Hif-1α protein levels by blocking Hif-1α protein synthesis, and continuous exposure to the compound was cytostatic. This compound inhibited phosphorylation of Akt, extracellular-regulated kinase 1/2, and stress-activated protein kinase/c-Jun-NH_2-kinase, which could be related to decreased Hif-1α synthesis and lead to a slowdown in cell proliferation. Hif-1α transcativation can be modulated by thioredoxin, which is a ubiquitously expressed small redox protein with a conserved catalytic site (47), that regulates the activity of enzymes such as apoptosis signal–regulating kinase-1 and increases the DNA binding of redox-sensitive transcription factors, which includes nuclear factor-κB, p53, and Hif-1α (31, 48–50). Inhibitors of Trx pathway, PX-12, and pleurotin developed by Powis et al. (30) have been shown to down-regulate hypoxia-induced increase and constitutive expression of Hif-1α and Hif-1α transcription factor activity. These compounds were also shown to be toxic following 16 hours of exposure followed by drug-free incubation for the remainder of the 72-hour incubation. Thioredoxin is required to regulate the activity of enzymes such as apoptosis signal-regulating kinase-1 (48); thus, inhibiting thioredoxin may sensitize cells to apoptosis. It is possible that DJ12, DJ15, and DJ30 may also inhibit enzymes upstream of Hif-1α, sensitizing cells to cell death. The 16-hour VEGF IC_{50} values for the three compounds were similar to the 72-hour viability IC_{50} values, which suggests that these compounds may inhibit regulatory pathways that regulate Hif-α activity with delayed effect on cell survival second.

Recently, a small molecule called chetomin was identified that disrupts the CH1 domain of p300 transcriptional coactivator and precludes its interaction with Hif-α, thereby attenuating hypoxia-inducible transcription in cell culture, as well *in vivo* in mice (36). However, blocking the CH1 domain of p300 may block other CH1-interacting transcriptional pathways.

We conclude that DJ12 is a novel inhibitor of Hif function. It is an effective *in vitro* drug against the Hif hypoxia pathway in breast, melanoma, and renal cancer cell lines. We have shown that DJ12 in MDA-468 breast cancer cell line leads to decreased Hif-1α transactivation, DNA binding, and expression of Hif-α target genes at RNA and protein level *in vitro*. However, long-term toxicity assay revealed that DJ12, DJ15, DJ30, and NSC-607097 were toxic in MDA-468 cells, which suggests that these compounds may have additional effects as seen for other compounds, or this may reflect inhibition of multiple Hif targets. The majority of new anti-Hif compounds have toxic/antiproliferative effects, which could be due to their nonspecific effects on cell signaling pathways. However, these pathways may regulate the hypoxia pathway indirectly, e.g., the above-mentioned thioredoxin pathway.

We plan to improve the potency of DJ12 through a structure-based approach. This will include screening a follow-up library in which different regions of DJ12 will be refined. Screening of a small library of 20 DJ12-like compounds has revealed six compounds with similar VEGF and cell viability profile as DJ12. As a result of such structure-activity relationship studies on DJ12 structure, it is expected that a new DJ12-like molecule will have a more potent inhibitory activity against the Hif-α pathway.

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