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Echinomycin, a Small-Molecule Inhibitor of Hypoxia-Inducible Factor-1 DNA-Binding Activity

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Abstract

The identification of small molecules that inhibit the sequence-specific binding of transcription factors to DNA is an attractive approach for regulation of gene expression. Hypoxia-inducible factor-1 (HIF-1) is a transcription factor that controls genes involved in glycolysis, angiogenesis, migration, and invasion, all of which are important for tumor progression and metastasis. To identify inhibitors of HIF-1 DNA-binding activity, we expressed truncated HIF-1α and HIF-1β proteins containing the basic-helix-loop-helix and PAS domains. Expressed recombinant HIF-1α and HIF-1β proteins induced a specific DNA-binding activity to a double-stranded oligonucleotide containing a canonical hypoxia-responsive element (HRE). One hundred twenty-eight compounds previously identified in a HIF-1–targeted cell-based high-throughput screen of the National Cancer Institute 140,000 small-molecule library were tested in a 96-well plate ELISA for inhibition of HIF-1 DNA-binding activity. One of the most potent compounds identified, echinomycin (NSC-13502), a small-molecule known to bind DNA in a sequence-specific fashion, was further investigated. Electrophoretic mobility shift assay experiments showed that NSC-13502 inhibited binding of HIF-1α and HIF-1β proteins to a HRE sequence but not binding of the corresponding proteins to activator protein-1 (AP-1) or nuclear factor-κB (NF-κB) consensus sequences. Interestingly, chromatin immunoprecipitation experiments showed that NSC-13502 specifically inhibited binding of HIF-1 to the HRE sequence contained in the vascular endothelial growth factor (VEGF) promoter but not binding of AP-1 or NF-κB to promoter regions of corresponding target genes. Accordingly, NSC-13502 inhibited hypoxic induction of luciferase in U251-HRE cells and VEGF mRNA expression in U251 cells. Our results indicate that it is possible to identify small molecules that inhibit HIF-1 DNA binding to endogenous promoters. (Cancer Res 2005; 65(19): 9047-55)

Introduction

Aberrant signaling pathways originating from oncogenic transformation, loss of function mutations, or dysregulation of receptor tyrosine kinases all converge on transcription factors to generate the cancer phenotype. Conceivably, there are much fewer transcription factors than upstream signaling networks that may be dysregulated in cancer cells and that may represent potential targets for interfering with their transcriptional program. Therefore, transcription factors may represent attractive targets for development of therapeutics (1). However, most of the activities of transcription factors are mediated by protein-protein interaction or binding to DNA in a sequence-specific fashion, both of which are conventionally considered challenging targets for development of small-molecule inhibitors (2).

Hypoxia-inducible factor-1 (HIF-1), a heterodimer composed of two subunits, HIF-1α and HIF-1β (also known as aryl hydrocarbon receptor nuclear translocator), mediates the transcriptional program to oxygen deprivation (3). Whereas HIF-1β is constitutively expressed in normoxic cells, HIF-1α protein levels are primarily regulated by intracellular oxygen concentrations (4), by a mechanism involving hydroxylation of two proline residues, recognition by the product of the *VHL* tumor suppressor gene, ubiquitylation, and proteasomal degradation (5). However, under normoxic conditions, HIF-1α levels and/or its transcriptional activity may be induced by alterations that inactivate tumor suppressor genes, such as *p53*, *PTEN*, and *VHL*, or activate oncogenes, such as *RAS* or *Src*, or by stimulation with growth factors, such as transforming growth factor-α, insulin-like growth factor-I/II, and epidermal growth factor (5–11).

HIF-1α and HIF-1β belong to the basic-helix-loop-helix (bHLH)-PAS family of transcription factors. The basic domain is responsible for sequence-specific DNA binding, whereas the HLH and the PAS domains mediate dimerization and stabilization of the complex on the DNA (12). The DNA-binding site recognized by HIF-1, known as hypoxia-responsive element (HRE), contains the core sequence 5′-R(A/G)CGTG-3′, which is present in promoters of HIF-1 target genes. HIF-1 activates the transcription of genes whose products are involved in crucial aspects of cancer biology, including cell survival, glycolysis, angiogenesis, migration, and invasion (5).

We report here that echinomycin (NSC-13502) inhibits HIF-1 DNA-binding activity. Interestingly, chromatin immunoprecipitation (ChIP) experiments indicated that NSC-13502 inhibited binding of HIF-1 to the endogenous HRE of the vascular endothelial growth factor (VEGF) promoter but not the binding of activator protein-1 (AP-1) or nuclear factor-κB (NF-κB) proteins to corresponding promoters. Accordingly, NSC-13502 inhibited HIF-1–dependent luciferase expression in U251-HRE cells and hypoxic induction of VEGF mRNA expression in U251 cells. Our data provide proof-of-principle that it is possible to identify small molecules that specifically inhibit HIF-1 DNA binding and raise the attractive possibility that this approach may lead to modulation of HIF-1–dependent gene expression.

Materials and Methods

Cell culture and reagents. U251 human glioma cells and MCF-7 cells were maintained in RPMI 1640 containing 5% fetal bovine serum and

Note: Supplementary data for this article are available at Cancer Research Online (http://cancerres.aacrjournals.org/).

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2 mmol/L glutamine at 37°C in a humidified incubator containing 21% O_2, 5% CO_2 in air (called normoxic conditions). U251-HRE cells (stably transfected with pGL2-TK-HRE) and U251-pGL3 cells (stably transfected with pGL3 vector), described previously (13), were maintained under the same conditions, except for the addition of 100 μg/mL G418. For treatment under hypoxic conditions, dish or plates were placed into a modular incubator chamber (Billups Rothenberg, Del Mar, CA), flushed for 20 minutes with a mixture of 1% O_2, 5% CO_2, and 94% N_2 (called hypoxic conditions), and then placed at 37°C in a humidified incubator. Sf9 insect cells were maintained at 28°C and ambient atmosphere in SF-900 II serum-free medium (Invitrogen, Carlsbad, CA). Compounds screened were from the Developmental Therapeutics Program, National Cancer Institute (NCI).

Recombinant proteins and nuclear extracts. The bHLH-PAS domain of the human HIF-1α cDNA (1,167 bp) was PCR amplified and cloned into pTriEx4-His tag expression vector. Protein was expressed in Sf9 insect cells with recombinant baculoviruses, which are prepared by cotransfecting Sf9 cells with pTriEx4-His tag expression vector and BacVector-3000 Triple Cut Virus DNA using Eufectin transfection reagent (Novagen, Madison, WI) as per manufacturer's protocol, and purified by affinity chromatography on nickel resin columns. The bHLH-PAS domain of the human HIF-1β cDNA (1,425 bp) was PCR amplified and cloned into pFlag1 expression vector (Sigma, St. Louis, MO). Protein was expressed in *Escherichia coli* DH5α cells and purified by M2 affinity chromatography. Recombinant p65 was purchased from ActiveMotif (Carlsbad, CA). Recombinant c-Fos/JunD proteins were kindly provided by Dr. Chuck Vinson [Center for Cancer Research (CCR), NCI, Bethesda, MD]. Preparation of nuclear extracts from U251 and MCF-7 cells was as described previously (13).

Hypoxia-inducible factor-1 DNA-binding ELISA. Biotin-labeled double-stranded DNA oligonucleotide (~30 pmol) encompassing a canonical HIF-1-binding site was added onto a streptavidin-immobilized ELISA plate (Pierce, Rockford, IL). Recombinant HIF-1α-bHLH-PAS (10 pmol) and HIF-1β-bHLH-PAS (6 pmol) were mixed in 50 μL of 1× buffer [25 mmol/L Tris-HCl (pH 7.6), 100 mmol/L KCl, 0.2 mmol/L EDTA, 20% glycerol, 5 mmol/L DTT]. After preincubation for 5 minutes at room temperature, protein mix was added to the plate and incubated at room temperature for 1 hour. The protein complex was detected by anti-His tag monoclonal antibody (Novagen) at a dilution of 1:2,000 and anti-mouse horseradish peroxidase–conjugated antibody (Sigma) at a dilution of 1:10,000. After incubating all wells with 100 μL TMB substrate solution (Sigma) for 10 minutes at room temperature, 0.5 mol/L H_2SO_4 (100 μL) was added and an absorbance was read at 450 nm with a reference wavelength of 655 nm. The sense strand sequences of the double-stranded oligonucleotides were wild-type (WT) HRE 5′-GTGCTACGTGCTGCCTAG-3′ and mutant HRE 5′-GTGCTAAAAGCTGCCTAG-3′. The biotin was labeled at the 5′ end of the sense strand. Oligonucleotide competition experiments were done with 50 pmol nonbiotinylated oligonucleotides. Compounds were screened at a concentration of 10 μmol/L.

Electrophoretic mobility shift assay. Recombinant HIF-1α-bHLH-PAS/HIF-1β-bHLH-PAS, NF-κB (p65), c-Fos/JunD, or nuclear extracts were used in electrophoretic mobility shift assay (EMSA). The double-stranded oligonucleotides encompassing the HIF-1-, NF-κB-, or AP-1-binding sites were labeled with [^{32}P]dCTP using the Klenow enzyme (Invitrogen). EMSA was done as described (13). Recombinant HIF-1α-bHLH-PAS (10 pmol) and HIF-1β-bHLH-PAS (6 pmol), 100 ng p65, or 10 pmol c-Fos and 10 pmol JunD, respectively, were mixed in 40 μL of 1× buffer [25 mmol/L Tris-HCl (pH 7.6), 100 mmol/L KCl, 0.2 mmol/L EDTA, 20% glycerol, 5 mmol/L DTT, 200 ng calf thymus DNA]. After preincubation for 5 minutes at room temperature, 1 μL labeled probe (5×10^4 cpm) was added and incubated on ice for 20 minutes. The sense strand sequences of the double-stranded oligonucleotide were WT HRE 5′-GTGCTACGTGCTGCCTAG-3′, mutant HRE 5′-GTGCTAAAAGCTGCCTAG-3′, NF-κB 5′-AGTTGAGGGGACTTTCCCAGGC-3′, AP-1 5′-CTAGTGATGAGTCAGCCGGATC-3′, WT Myc/Max 5′-AGTTGACCACGTGGTCTGGG-3′, and mutant Myc/Max 5′-AGTTGACTAAAAGGTCTGGG-3′. Competition experiments were done with unlabeled WT or mutant oligonucleotides (100-fold excess). Monoclonal anti-HIF-1α antibody (NB100-105) from Novus (Littleton, CO) and polyclonal anti-HIF-1α antibody (H-206) from Santa Cruz Biotechnology (Santa Cruz, CA) were used in supershift assay.

Chromatin immunoprecipitation assay. ChIP-IT kit was purchased from ActiveMotif. ChIP assay was done according to the manufacturer's protocol. Anti-HIF-1α monoclonal antibody was from Novus and anti-c-Fos polyclonal antibody (H-125) and anti-p65 polyclonal antibody (C-20) were from Santa Cruz Biotechnology. For immunoprecipitation, 2 μg of each corresponding antibody were used. PCR primer sets were the following: VEGF promoter, forward 5′-CCTTTGGGTTTTGCCAGA-3′ and reverse 5′-CCAAGTTTGTGGAGCTGA-3′; cyclin D1 promoter, forward 5′-CTACAC-CCCCAACAAAACCA-3′ and reverse 5′-TAACCGGGAGAAACACACCT-3′; and intercellular adhesion molecule-1 (ICAM-1) promoter, forward 5′-CGTGATTCAAGCTTAGCCTG-3′ and reverse 5′-TTATTTCCGGACTGA-CAGGG-3′. For each PCR reaction, primer concentration was 10 pmol/20 μL. Following 32 to 35 cycles of amplification, the PCR products were run on a 3.0% agarose gel and analyzed by ethidium bromide staining.

Western blot. Cells were washed with ice-cold PBS and lysed by incubation on ice in lysis buffer containing 50 mmol/L Tris-HCl (pH 7.4), 1% NP40, 150 mmol/L NaCl, and complete protease inhibitor cocktail (Roche Diagnostics, Indianapolis, IN). Lysates were centrifuged at 13,000 rpm for 5 minutes at 4°C and the supernatant was used for Western blotting done as described previously (13). Polyclonal anti-HIF-1α (H-206) antibody and anti–cyclin D1 antibody (C-20) were from Santa Cruz Biotechnology. Monoclonal anti-actin (MAB1501) antibodies were purchased from Chemicon International (Temecula, CA). HIF-1α and cyclin D1 were detected using a 1:200 dilution of the specific antibody, whereas anti-actin antibody was diluted 1:3,000.

DNA thermal melting analysis. Thermal transition curves were obtained with a Perkin-Elmer Lambda (Boston, MA) 20 spectrophotometer using a DBP Peltier System. Absorption changes at 260 nm were collected as a function of temperature. Oligonucleotides were prepared at 0.5 μmol/L in the presence or absence of 1 μmol/L small molecule in 10 mmol/L Tris (pH 8.0), 1 mmol/L EDTA. DMSO concentrations were 0.1% and had no effect on the T_m of the oligonucleotides. Melting temperatures were calculated using TempLab software provided by Perkin-Elmer. The sense strand sequences of the double-stranded oligonucleotides used in this assay were HRE 5′-TACGTGCTTACGTGCTTACGTGCT-3′, NF-κB-binding site 5′-AGGGGACTTTCCCAGGGGACTTTCCC-3′, and AP-1-binding site 5′-AGTGATGAGTCAGAGTGATGAGTCAG-3′.

Luciferase assay. U251-HRE cells were seeded onto 24-well plate the day before treatment. Luciferase assay was done as described previously (13). Luminescence reading was normalized by protein concentration and expressed as fold relative to the reference sample (untreated normoxic control). Cytotoxicity was assayed using the sulforhodamine B (SRB) assay as described previously (14).

Real-time PCR. Total RNA from U251 cells was obtained using RNA Mini kit (Qiagen, Valencia, CA). Total RNA (1 μg) was used to perform reverse transcription-PCR (RT-PCR) using RT-PCR kit (PE Biosystems, Foster City, CA). To measure human VEGF and HIF-1α mRNA expression, real-time PCR was done as described previously (13). The following primers and probes were used: human VEGF, forward 5′-TACCTCCACCATGCCAAGTG-3′ and reverse 5′-ATGATTCTGCCCTCCTCCTTC-3′; VEGF probe, 5′-FAM-TCCCAGGCTGCACCCATGGC-TAMRA-3′; and human HIF-1α, forward 5′-GCAGCTACTACATCACTTTCTT-3′ and reverse 5′-CAGCAGTCTACATGC-TAAATCA-3′. Detection of 18S rRNA, used as internal control, was pre-formed using premixed reagents from Applied Biosystems (Foster City, CA). Detection of VEGF and 18S rRNA was done using TaqMan Universal PCR Master Mix (Applied Biosystems), whereas HIF-1α detection was done using SYBR Green PCR Master Mix (Applied Biosystems).

Transcriptional profiling of the effects of hypoxia and echinomycin treatment on U251 cells. Human 22K oligonucleotide microarrays (Operon-22K-oligo-V2 from the NCI/CCR Microarray Center) were used according to protocols published on the mAdB homepage.[3] Briefly, logarithmically growing U251 cells were treated with 20 nmol/L NSC-13502 for 20 hours under either normoxia or hypoxia. Total RNA was extracted from these samples using the RNeasy Mini kit (Qiagen); then,

[3] http://nciarray.nci.nih.gov

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equal amounts (20 μg) were reverse transcribed and amino-allyl modified dUTP was incorporated into control and drug treated samples using the Fairplay kit (Stratagene, La Jolla, CA). Each cDNA sample was then chemically coupled to a Cy3 (control) or Cy5 (treated) fluorescently labeled dye and purified, the two probes were combined, filtered, and blocked, and the remaining samples were transferred to a prehybridized glass array under a coverslip. Arrays were hybridized at 42°C for 16 hours, washed thrice, and dried. Fluorescence was read on a GenePix 4100A microarray scanner at a wavelength of 635 nm for the Cy5 (pseudo-colored red) and 532 nm for the Cy3 samples (pseudo-colored green). Data were analyzed through GenePix Pro 4.1 software; then, data and image files were uploaded to the NCI/CCR Microarray Center mAdB Gateway for storage, analysis, and multiple array comparisons.

Results

Expression of recombinant hypoxia-inducible factor-1α and hypoxia-inducible factor-1β proteins and hypoxia-inducible factor-1 DNA-binding ELISA screening. To develop assays for the identification of small-molecule inhibitors of HIF-1 DNA-binding activity, we expressed recombinant HIF-1α-bHLH-PAS-His (HIF-1α-389) and Flag-HIF-1β-bHLH-PAS (HIF-1β-475) proteins as described in Materials and Methods (Fig. 1*A*). EMSA experiments showed that HIF-1α-389 and HIF-1β-475 recombinant proteins mediated a specific DNA-binding activity to an oligonucleotide containing the canonical HIF-1-binding site (HRE), which was competed for by addition of excess unlabeled WT, but not mutant, oligonucleotide (Fig. 1*B*). To screen large number of compounds, we tested HIF-1α-389 and HIF-1β-475 recombinant proteins in a DNA-binding ELISA using a biotinylated HRE bound to streptavidin plates. Similar to results obtained in EMSA, HIF-1α-389 and HIF-1β-475 mediated specific DNA-binding activity that was competed for by addition of excess nonbiotinylated WT, but not mutated, oligonucleotide (Fig. 1*C*). One hundred twenty-eight active compounds from a U251-HRE-based high-throughput screen of the NCI 140,000 small-molecule library were then tested using the HIF-1 DNA-binding ELISA. Twenty of 128 compounds inhibited HIF-1β-389 and HIF-1β-475 DNA-binding activity by >50% relative to untreated control at the 10 μmol/L concentration (data not shown). These compounds were further validated in EMSA using HIF-1α-389 and HIF-1β-475 (HIF-1) proteins; to control for specificity, c-Fos and JunD (AP-1) and p65 (NF-κB) binding to the corresponding DNA oligonucleotide consensus sequences was also tested. EMSA results showed that all 20 compounds inhibited HIF-1 DNA-binding activity, consistent with results obtained in ELISA. However, 12 of 20 compounds also inhibited either AP-1 or NF-κB DNA binding or both, suggesting that these compounds were nonspecific inhibitors of protein DNA binding (data not shown). Eight compounds showed a relative specificity for inhibition of HIF-1 DNA binding (Supplementary Fig. S1).

Identification of echinomycin as a potent inhibitor of hypoxia-inducible factor-1 DNA-binding activity. Figure 2*A* shows the chemical structure of NSC-13502, one of the most potent compounds identified in the HIF-1 cell-based high-throughput screen and further validated using DNA-binding assays. ELISA experiments (Fig. 2*B*) showed that NSC-13502 potently inhibited HIF-1α-389 and HIF-1β-475 DNA-binding activity in a dose-dependent fashion, with almost complete inhibition at 320 nmol/L. Similar results were obtained in EMSA where NSC-13502 inhibited in a dose-specific fashion DNA-binding activity induced by HIF-1α-389 and HIF-1β-475 recombinant proteins (Fig. 2*C-a*). More importantly, NSC-13502 up to 10 μmol/L did not inhibit DNA-binding activities induced by c-Fos and JunD to the corresponding AP-1 DNA-binding sequence (Fig. 2*C-b*) or the binding of p65 to the NF-κB consensus sequence (Fig. 2*C-c*). These results indicate that NSC-13502 specifically inhibited HIF-1–dependent, but not AP-1– or NF-κB–dependent, DNA-binding activity. Interestingly, NSC-13502 was originally discovered as a sequence-specific DNA-binding agent (15), whose sequence specificity encompasses the core sequence (5′-CGTG-3′) of HRE consensus sequence 5′-R(A/G)CGTG-3′ (12). To further define the mechanism by which NSC-13502 inhibited HIF-1 DNA-binding activity, we first characterized the DNA-binding activities to a canonical HRE in U251 cells cultured under normoxic or hypoxic conditions. As shown in Fig. 2*D-a*, hypoxia induced a DNA-binding activity to the HRE (*lane 2*) that was supershifted (*lane 3*) or abrogated (*lane 4*) by two different anti-HIF-1α antibodies and was competed for by excess of

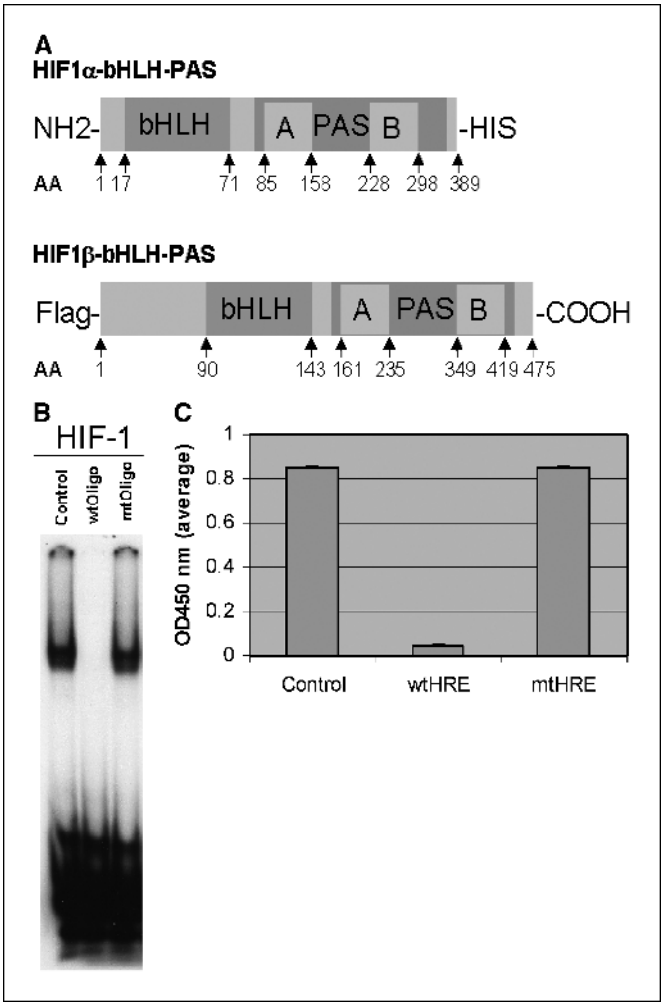

Figure 1. *A,* schematic representation of recombinant HIF-1α-bHLH-PAS and HIF-1β-bHLH-PAS proteins. HIF-1α-389 (amino acids 1-389) protein fused to a His tag and HIF-1β-475 (amino acids 1-475) protein fused to a Flag tag were expressed as described in Materials and Methods. *B,* EMSA: DNA-binding reactions were carried out by adding recombinant HIF-1α-389 and HIF-1β-475 in the presence of ^{32}P-labeled oligonucleotide containing a canonical HRE. Competition experiments were done using 100-fold molar excess of unlabeled oligonucleotide containing either WT or mutated HRE. *C,* ELISA: A biotinylated double-stranded oligonucleotide containing a canonical HRE was bound to streptavidin-coated plates. Recombinant HIF-1α-389 and HIF-1β-475 were added to individual wells as described in Materials and Methods. For competition experiments, ~10-fold excess of nonbiotinylated oligonucleotide, either WT or containing a mutated HRE, was added to the wells before addition of recombinant proteins.

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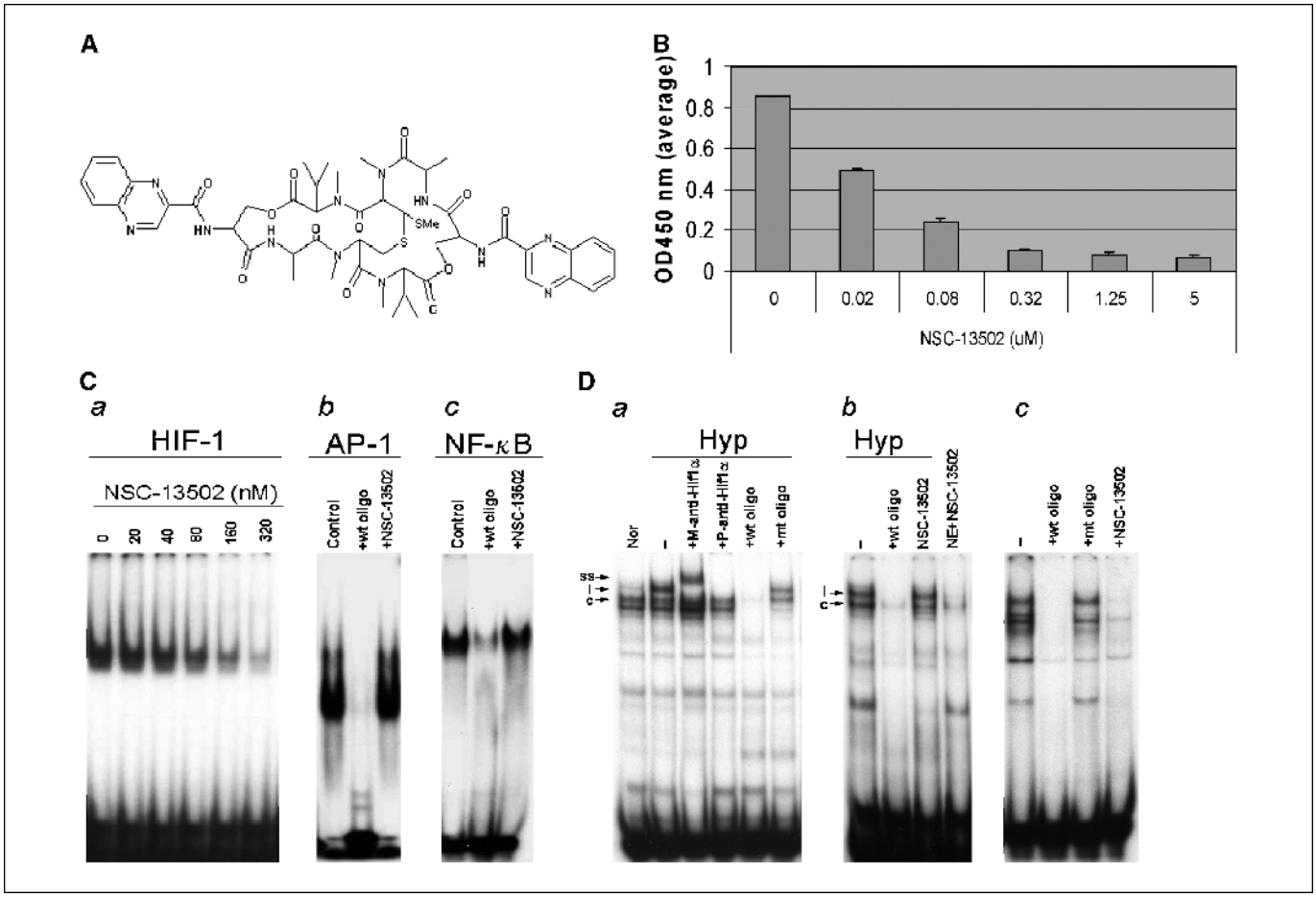

Figure 2. NSC-13502 specifically inhibits HIF-1 DNA binding to the HRE. *A,* chemical structure of NSC-13502. *B,* ELISA: As described in Materials and Methods, recombinant HIF-1α-389 and HIF-1β-475 were added to individual wells in the presence of increasing concentrations of NSC-13502. *C,* EMSA: *a,* as described in Materials and Methods, recombinant HIF-1α-389 and HIF-1β-475 were added to DNA-binding reactions in the presence of increasing concentrations of NSC-13502 as indicated. The oligonucleotide used in this experiment contained the 5′-ACGTG-3′ consensus sequence. Similar results were obtained using an oligonucleotide containing the 5′-GCGTG-3′ binding site (data not shown). *b,* EMSA was carried out using recombinant c-Fos and JunD proteins and an oligonucleotide containing a canonical AP-1-binding site, as described in Materials and Methods, in the presence of excess unlabeled AP-1 oligonucleotide (*lane 2*) or NSC-13502 (10 μmol/L) directly added to the binding reaction before addition of recombinant proteins. *c,* EMSA was done using recombinant p65 protein and a ^{32}P-labeled oligonucleotide containing a canonical NF-κB-binding site in the presence of excess unlabeled oligonucleotide (*lane 2*) or NSC-13502 (10 μmol/L) directly added to the binding reaction. *D,* EMSA: *a,* EMSA was done using nuclear extracts harvested from U251 cells cultured under normoxia (*lane 1*) or hypoxic (*lanes 2-6*) conditions for 16 hours. Monoclonal (*lane 3*) or polyclonal (*lane 4*) anti-HIF-1α antibodies were used for supershift assay. Excess WT HRE (*lane 5*) or mutant HRE (*lane 6*) was added to the binding reaction for competition assay. *b,* EMSA was done using nuclear extracts harvested from U251 cells cultured under hypoxic conditions for 16 hours in the absence (*lane 1*) or presence (*lane 3*) of NSC-13502 (1 μmol/L). Excess WT HRE (*lane 2*) or 1 μmol/L NSC-13502 (*lane 4*) was added to the binding reaction done using nuclear extracts from hypoxia-treated cells. *c,* EMSA was done using nuclear extracts harvested from untreated MCF-7 cells. Excess oligonucleotide containing a WT (*lane 2*) or mutant (*lane 3*) E-box consensus sequence was added to the binding reaction for competition assay. *Lane 4,* NSC-13502 (1 μmol/L) was added to the binding reaction. *c,* constitutive; *I,* inducible; *ss,* supershift.

unlabeled WT (*lane 5*), but not mutated (*lane 6*), oligonucleotide. Next, we tested the ability of NSC-13502 to inhibit HIF-1 DNA-binding activities in nuclear extracts isolated from cells cultured under hypoxic conditions. Treatment of hypoxic U251 cells with NSC-13502 had a minimal effect on both constitutive and inducible HIF-1 DNA-binding activities detected in nuclear extracts (Fig. 2*D-b*, *lane 3*). In contrast, addition of NSC-13502 to the binding reaction *in vitro* completely abrogated HIF-1-dependent DNA-binding activities detected in nuclear extracts from hypoxic U251 cells (Fig. 2*D-b*, *lane 4*). These results show that NSC-13502 inhibits DNA binding of endogenous full-length HIF-1α and HIF-1β proteins and are consistent with the possibility that NSC-13502 inhibits HIF-1 DNA binding by interacting with DNA and not by interfering with protein-protein interaction. The core sequence of the HRE (5′-CGTG-3′) is also present in the E-box (5′-CACGTG-3′), which is the DNA-binding site of the Myc/Max complex, raising the possibility that NSC-13502 might also inhibit the binding of nuclear proteins to the E-box. To test this possibility, we did EMSA experiments using nuclear extracts harvested from untreated MCF-7 cells and an oligonucleotide containing the E-box. As shown in Fig. 2*D-c*, MCF-7 cells showed constitutive DNA-binding activities (*lane 1*) to the E-box that were competed for by excess of unlabeled WT (*lane 2*), but not mutant (*lane 3*), oligonucleotide. Interestingly, addition of NSC-13502 to the DNA-binding reaction completely abrogated binding of nuclear proteins to the DNA (*lane 4*), providing further evidence of the sequence specificity of NSC-13502.

Echinomycin inhibits hypoxia-inducible factor-1 binding to the hypoxia-responsive element of endogenous vascular endothelial growth factor promoter. Results shown thus far indicate that NSC-13502 inhibits HIF-1, but not AP-1 or NF-κB, DNA-binding activity *in vitro* by binding DNA in a sequence-specific fashion. If this assumption were correct, a ChIP assay would show

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whether NSC-13502 inhibited endogenous HIF-1 DNA-binding activity in cultured cells. To address this question, U251 cells were treated with hypoxia and desferrioxamine for 6 hours to induce maximal HIF-1 DNA-binding activity to cognate DNA-binding sites of endogenous promoters in the absence or presence of NSC-13502 (0.5 μmol/L). As shown in Fig. 3*A*, HIF-1 DNA-binding activity to the HRE present at positions −947 to −939 (5′-TACGTG-3′) of the VEGF promoter was detected in cells treated with hypoxia and desferrioxamine. Treatment of U251 cells with NSC-13502 completely abrogated HIF-1 DNA-binding activity to the VEGF promoter. Importantly, NSC-13502 did not affect the binding of AP-1 (c-Fos) to the cyclin D1 promoter or the binding of NF-κB (p65) to the ICAM-1 promoter, transcription factors that were constitutively present in untreated U251 cells. These results clearly show that NSC-13502 specifically inhibits HIF-1, but not AP-1 or NF-κB, binding activity to endogenous promoters in cultured cells.

Echinomycin does not inhibit hypoxia-inducible factor-1α protein accumulation. To rule out the possibility that the inhibition of HIF-1 DNA-binding activity was dependent on down-regulation of HIF-1α protein levels, we did Western blotting analysis in U251 cells treated under normoxic or hypoxic conditions in the presence or absence of NSC-13502 (0.5 μmol/L). As shown in Fig. 3*B*, hypoxia induced HIF-1α protein accumulation, which was slightly increased by addition of NSC-13502. In addition, cyclin D1 protein levels were not affected by treatment under hypoxia or with NSC-13502, consistent with data obtained in the ChIP assay. In conclusion, NSC-13502 does not affect HIF-1α protein levels and does not have a general effect on other proteins.

DNA melting temperature analysis. NSC-13502 is a small molecule known to bind DNA in a sequence-specific fashion (15). Intercalation of small molecules into the double helix is known to increase the helix melting temperature (16, 17). The extinction coefficient of DNA bases at 260 nm in the double-helical form is much less than in the single-stranded form; thus, melting of the helix leads to an increase in the absorption at this wavelength. The helix to coil transition temperature can be determined by monitoring the absorbance of the DNA bases at 260 nm as a function of temperature. To determine whether compound NSC-13502 binds to DNA in a relatively specific manner to inhibit HIF-1 protein/DNA–binding activity, we did DNA melting temperature analysis of the double-stranded HRE, AP-1 and NF-κB oligonucleotides, in the absence or presence of NSC-13502 (Supplementary Fig. S2). The melting temperature (T_m) of HRE DNA was increased from 51.5°C to 65°C (26.2% increase), whereas only a 5.5°C increase for NF-κB binding and a 5°C increase for AP-1-binding oligonucleotides were observed, indicating that NSC-13502 exhibits a higher affinity for HRE core sequence than AP-1- or NF-κB-binding core sequences. These results were further validated by analysis of the activity of NSC-13502 on AP-1 and HRE hairpin oligonucleotides using the ethidium bromide displacement assay. NSC-13502 caused ~50% displacement of ethidium bromide on HRE oligonucleotide at 3 μmol/L, whereas no significant influence on AP-1 oligonucleotide was observed even at a concentration of 10 μmol/L (data not shown).

Echinomycin inhibits hypoxia-inducible factor-1–dependent luciferase expression in U251-HRE cells. We have described previously U251-HRE and U251-pGL3 cells, which express the

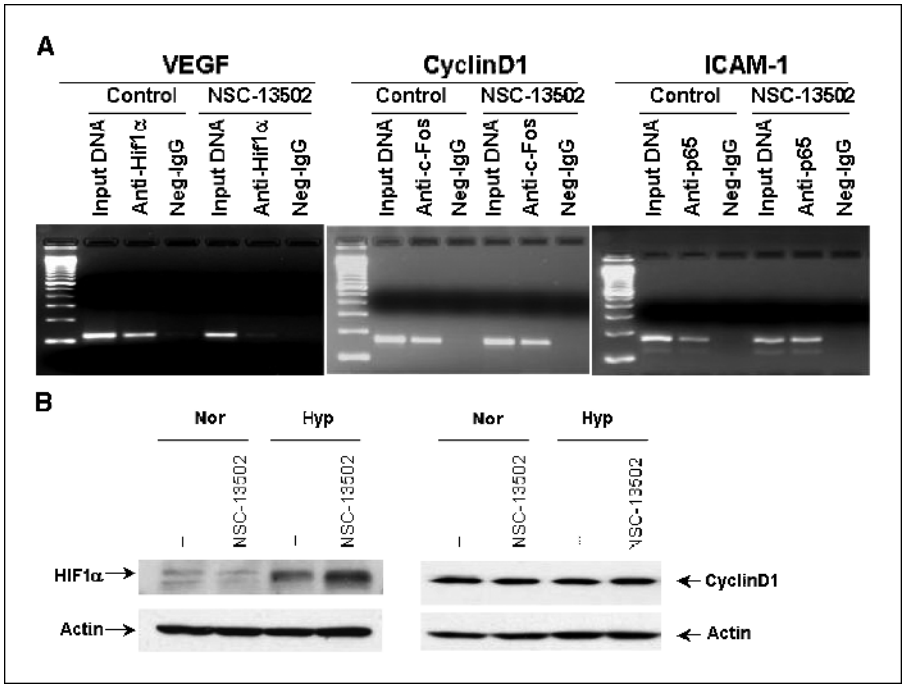

Figure 3. NSC-13502 inhibits HIF-1 binding to the HRE of the VEGF promoter in cultured cells. *A,* ChIP assay: For VEGF promoter, U251 cells were treated with hypoxia plus desferrioxamine (400 μmol/L) in the presence or absence of NSC-13502 (0.5 μmol/L) for 6 hours. ChIP assay was done as described in Materials and Methods using an anti-HIF-1α or irrelevant isotype-matched control antibody, respectively. Input DNA was measured before performing immunoprecipitation and the sample was then divided between relevant and irrelevant antibodies. PCR fragment of 135 bp from the VEGF promoter is indicated. For cyclin D1 promoter, U251 cells were cultured under normoxic conditions for 6 hours in the absence or presence of NSC-13502 (0.5 μmol/L). ChIP was done using anti-c-Fos or isotype-matched irrelevant antibodies, respectively. PCR fragment of 163 bp from the cyclin D1 promoter is indicated. For ICAM-1 promoter, U251 cells were cultured under normoxic conditions for 6 hours in the absence or presence of NSC-13502 (0.5 μmol/L). ChIP was done using anti-p65 or isotype-matched irrelevant antibodies, respectively. PCR fragment of 176 bp from the ICAM-1 promoter is indicated. *B,* Western blot: U251 cells were treated under normoxic (*Nor*) or hypoxic (*Hyp*) conditions for 16 hours in the absence or presence of NSC-13502 (0.5 μmol/L). Total cell lysates were prepared as described in Materials and Methods. Immunoblotting was done using polyclonal anti-HIF-1α and polyclonal anti–cyclin D1 antibodies, respectively. Actin was assessed on the same membrane as internal control.

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luciferase reporter gene under control of HIF-1 or SV40 promoters, respectively (13). To further define the EC_{50} of NSC-13502 in inhibiting HIF-1–dependent transcriptional activity, U251-HRE and U251-pGL3 cells were treated under normoxic or hypoxic conditions in the presence or absence of increasing concentrations of NSC-13502. As shown in Fig. 4*A*, NSC-13502 very potently inhibited hypoxic induction of luciferase expression in U251-HRE in a dose-dependent fashion with an EC_{50} of ~1.2 nmol/L. In contrast, NSC-13502 did not significantly affect luciferase expression in the U251-pGL3 control cell line under either normoxic or hypoxic conditions (Fig. 4*B*). To test whether inhibition of luciferase expression in U251-HRE was reversible, cells were treated with NSC-13502 at 5, 10, and 20 nmol/L for 4 hours and then washed and incubated under normoxic or hypoxic conditions for 36 hours. As shown in Fig. 4*C*, 94%, 80%, and 42% of hypoxia-induced luciferase expression was restored in cells in which NSC-13502 was washed out after 4-hour treatment at 5, 10, and 20 nmol/L, respectively, indicating that the DNA-binding activity of NSC-13502 is in fact reversible. To determine whether NSC-13502 had any effect on cell viability at the concentrations used, we did a SRB assay in U251-HRE cells exposed to increasing concentrations of NSC-13502. The IC_{50} under normoxic or hypoxic conditions at 24 hours was >5 μmol/L (data not shown), indicating that NSC-13502 did not have a significant effect on cell viability under our experimental conditions.

Echinomycin inhibits hypoxic induction of vascular endothelial growth factor mRNA expression. To investigate the effects of NSC-13502 on VEGF mRNA expression, U251 cells were treated under normoxic or hypoxic conditions in the absence or presence of increasing concentrations of NSC-13502 (0-10 nmol/L) for 16 hours. As shown in Fig. 5*A*, hypoxia induced a 5-fold increase in VEGF mRNA expression, which was significantly ($P < 0.01$) inhibited by addition of NSC-13502 in a dose-dependent fashion. In contrast, HIF-1α mRNA expression, which was slightly decreased under hypoxic conditions relative to normoxia, was not affected by NSC-13502 (data not shown). To further investigate the global effect of NSC-13502 on gene expression, we did transcriptional profiling analysis using NCI human microarray sets, which contain oligonucleotides representing ≥11,000 genes. U251 cells were treated under normoxia or hypoxia in the presence of NSC-13502 (20 nmol/L) for 20 hours. Overall, we found that more genes were down-regulated than up-regulated, as

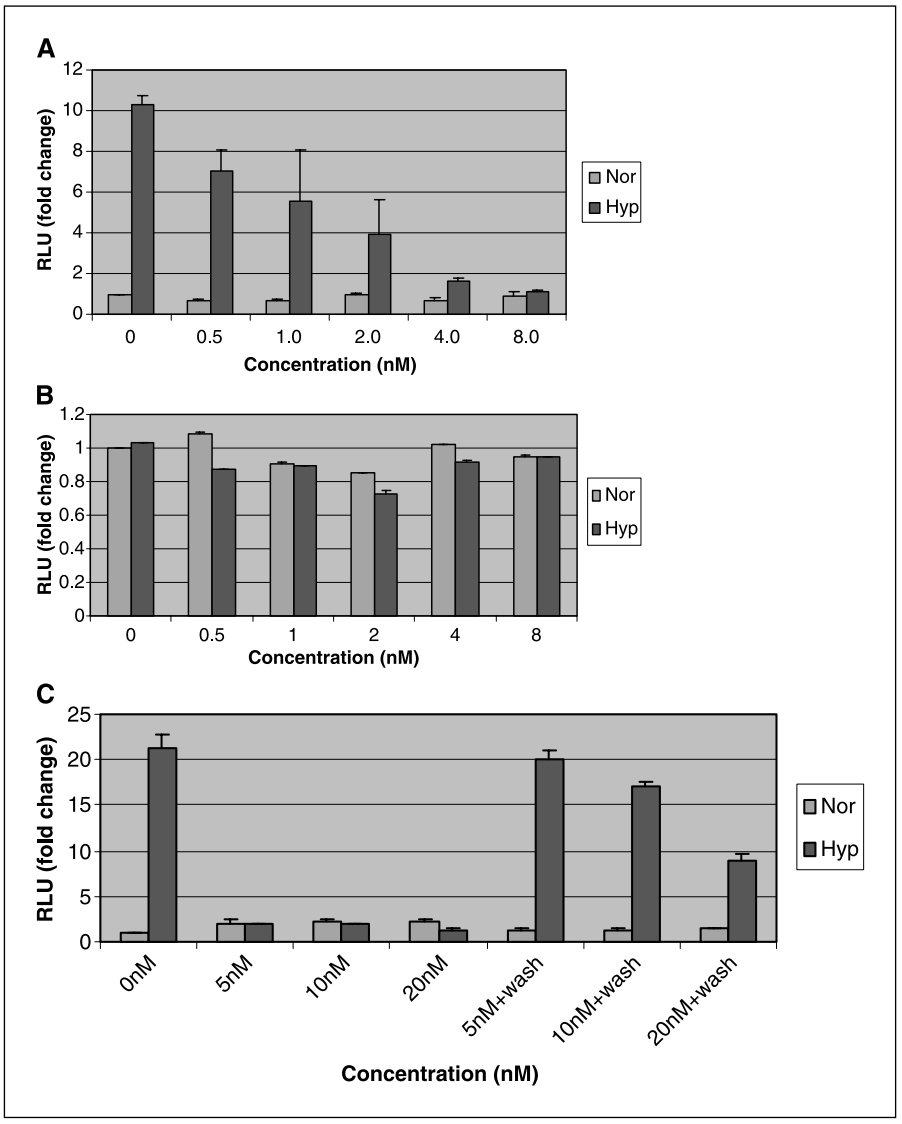

Figure 4. NSC-13502 inhibits hypoxic induction of luciferase expression in U251-HRE cells. U251-HRE (*A*) or U251-pGL3 cells (*B*) were seeded (2×10^5 per well) in 24-well plates and incubated under normoxic or hypoxic conditions for 16 hours in the presence or absence of the indicated concentrations of NSC-13502; then, expression of luciferase was measured. *C,* U251-HRE cells (1×10^5 per well) were seeded in 24-well plates and treated with the indicated concentrations of NSC-13502 for 4 hours. Cells were then washed thrice with medium and incubated under normoxic or hypoxic conditions in the presence or absence of the indicated concentrations of NSC-13502 for 36 hours. Cell lysates were harvested and luciferase expression was measured. Results are expressed as fold change relative to untreated cells cultured under normoxia condition (equal to 1) after normalization for protein concentration.

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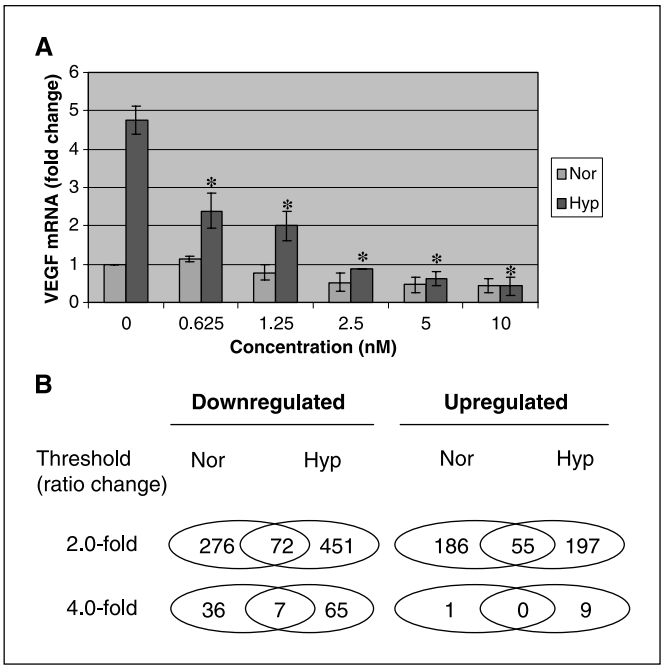

Figure 5. Effect of NSC-13502 on gene expression in U251 cells. *A,* VEGF mRNA expression: U251 cells were cultured under normoxic or hypoxic conditions in the absence or presence of the indicated concentrations of NSC-13502 for 16 hours. Total RNA was harvested and analyzed for human VEGF mRNA expression using real-time PCR as described in Materials and Methods. 18S rRNA was tested in parallel as internal control for input RNA. Results are expressed as fold increase relative to mRNA levels of untreated cells cultured under normoxic conditions (equal to 1). *Columns,* average of four independent experiments; *bars,* SE. *, $P < 0.01$, one-way ANOVA. *B,* microarray analysis: U251 cells were cultured under normoxic or hypoxic conditions for 20 hours in the absence or presence of NSC-13502 (20 nmol/L). Total RNA was harvested and processed as described in Materials and Methods. Data were analyzed through GenePix Pro 4.1 software; then, data and image files were uploaded to the NCI/CCR Microarray Center mAdB Gateway for storage, analysis, and multiple array comparisons. The numbers outside the intersections represent genes affected under hypoxia or normoxia; the numbers inside the intersections represent genes affected under both hypoxia and normoxia.

one might have predicted. In particular, 276 and 451 genes were down-regulated by ≥2-fold under normoxic or hypoxic conditions, respectively, in the presence of NSC-13502 (Fig. 5*B*). In contrast, only 186 genes were up-regulated under normoxia and 197 under hypoxia. Fewer genes were changed by ≥4-fold, suggesting that NSC-13502 has a limited effect on global gene expression (Fig. 5*B*). Notably, more genes were down-regulated under hypoxic than normoxic conditions (451 versus 276 by ≥2-fold and 65 versus 36 by ≥4-fold), consistent with the possibility that NSC-13502 inhibited preferentially HIF-1–dependent gene expression. Indeed, we found that several known HIF-1–inducible genes present on the array, which were up-regulated under hypoxic conditions, were also significantly decreased in the presence of NSC-13502. In particular, 17 of 109 genes induced by hypoxia by >2.5-fold could be identified as HIF-1–inducible genes based on published results. Fourteen of the 17 HIF-1–inducible genes were significantly inhibited by NSC-13502 by >3-fold (Table 1). Moreover, 10 known HIF-1–inducible genes were identified among the 65 genes inhibited >4-fold by NSC-13502 under hypoxic conditions. In conclusion, our results show that expression of a limited number of genes is affected by treatment of U251 cells with NSC-13502 and they are consistent with a predictable pattern of inhibition of known HIF-1–inducible genes.

Discussion

HIF-1α is the downstream mediator of hypoxic and nonhypoxic signaling pathways, which play a crucial role in cancer progression. HIF-1 induces expression of genes whose products are involved in cancer cells survival, glycolysis, angiogenesis, migration, and invasion (5). Notably, HIF-1α is overexpressed in human cancers due to hypoxia-dependent and/or hypoxia-independent pathways (6, 18, 19), and overexpression of HIF-1α has been implicated as a poor prognostic indicator in a variety of tumors (20–23). Therefore, HIF-1 is an attractive target for cancer therapy (5, 24, 25).

Several approaches have been used to inhibit HIF-1α expression and/or activity: antisense or small interfering RNA strategies (26, 27), inhibition of proteins that modulate HIF-1 activity (28–31), signal transduction pathways involved in HIF-1α activation (11, 32, 33), microtubules (34), topoisomerase I (35), or mechanisms not clearly defined (36, 37). Our group has recently shown that topotecan, a topoisomerase I poison, inhibits HIF-1α protein accumulation independently of DNA replication-mediated DNA damage, suggesting a distinct mechanism of action from the cytotoxic effects (35). More importantly, we have shown that topotecan administered on a chronic schedule inhibits HIF-1α protein accumulation, angiogenesis, and tumor growth in U251-HRE human glioblastoma xenografts (38). Most of the agents described above affect targets that are also involved in other biological functions, which will ultimately determine their therapeutic activity, and they can be defined nonselective inhibitors of HIF-1. Although these agents might be useful in the clinical setting as HIF-1 inhibitors, it remains difficult to clearly define the contribution of HIF-1 inhibition to their therapeutic activity and to establish how they should be used in the clinic to inhibit HIF-1.

In contrast, a selective inhibitor would target a specific step of HIF-1 activation pathway. Selective inhibitors would be ideal tools for probing the HIF-1 pathway and to establish the therapeutic potential of HIF-1 inhibition. Several domains of HIF-1 might be suitable targets for the development of selective HIF-1 inhibitors: domains involved in dimerization with HIF-1β, in the recruitment of coactivators, which are required for maximal transcriptional activity, or binding to DNA, which is dependent on sequence specificity. Each of these potential targets has been exploited to different extents.

One approach has been to interfere with the interaction between the C-TAD of HIF-1α and the CH1 domain of p300, a coactivator required for maximal transcriptional activity of HIF-1 (39). This interaction, which is at least in part dependent on the hydroxylation of an asparagine residue at position 802, has been targeted with chetomin, a small molecule that showed therapeutic activity in HCT116 and PC3 xenograft models (40). This approach, although attractive, is limited by the unstructured conformation of free HIF-1α C-TAD (41), which makes more problematic inhibiting its interaction with p300, and by concerns on the potential lack of specificity of an active agent.

Synthetic polyamides have been designed that interfere in a sequence-specific fashion with transcription factors to modulate their functional activity. Hairpin polyamides and polyamide-acridine conjugates have been shown to inhibit protein-DNA complexes in some cases (42, 43). Recently, a synthetic pyrrole-imidazole polyamide targeting the HRE site in the VEGF promoter has been described, which was designed to bind to the DNA sequence 5′-*W*T*W*CG*W*-3′ (where *W* = A or T; ref. 44).

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Table 1. Selected list of HIF-1-inducible genes down-regulated by NSC-13502 (20 nmol/L) in U251 cells cultured under hypoxia conditions

Genbank accession no.	Annotated genes	HRE	Log_2 value of ratio change	
			Hypoxia	NSC-13502 + hypoxia
AF022375	*VEGF*	TACGTG	1.6	−2.8
NM_000598	*IGFBP3*	ND*	4.0	−3.9
X60673	*AK3*	TGCGTG	2.1	−3.1
AY886764	*BNIP3*	CG/ACGTG	1.9	−3.6
NM_004331	*BNIP3L*	ND	1.5	−1.6
NM_019058	*DDIT4*	TACGTG	2.9	−4.4
NM_014367	*E2IG5*	GGCGTG	2.2	−3.1
NM_001956	*EDN2*	ND	1.5	−2.2
NM_000158	*GBE1*	ND	1.7	−3.3
NM_006096	*NDRG1*	ND	3.8	−4.4
NM_004567	*PFKFB4*	CGCGTG	1.8	−2.8
NM_001216	*CA9*	TACGTG	1.5	−2.2
NM_001124	*ADM*	AACGTG	2.3	−2.7
BC093742	*HIG2*	ND	3.3	−4.6

*ND, not determined.

This polyamide blocked HIF-1 DNA binding to the HRE of the VEGF promoter *in vitro* and inhibited VEGF mRNA expression and protein levels in cultured HeLa cells. Although the design of synthetic polyamides might offer a higher sequence-based specificity, their development as therapeutics remains to be further established.

Conceptually, a small molecule with favorable pharmacologic properties would be more attractive to inhibit HIF-1 DNA binding, although the identification of a sequence-specific compound that interferes with DNA binding of transcription factors remains elusive. We provide here proof-of-principle that NSC-13502 can in fact inhibit, in a sequence-specific fashion, the binding of HIF-1 to its cognate DNA-binding sequence.

NSC-13502, a cyclic peptide of the family of quinoxaline antibiotics that was originally isolated from *Streptomyces echinatus* (45), is a small molecule known to bind DNA in a sequence-specific fashion. The strong binding sites for NSC-13502 contain the central 2-bp sequence 5′-CG-3′, and the key recognition elements for NSC-13502 are contained in the sequences 5′-ACGT-3′ and 5′-TCGT-3′ (15). In EMSA, NSC-13502 showed a significant degree of specificity between inhibiting binding of HIF-1 to the HRE, which contains 5′-ACGT-3′ in its core sequence, and unrelated sequences recognized by the AP-1 or NF-κB family of transcription factors. Although the evidence provided by EMSA experiments was encouraging, it was far from proving that this compound retained the ability to inhibit endogenous HIF-1α and HIF-1β proteins and more importantly to block the binding of HIF-1 heterodimer to endogenous HRE-containing promoters. Two findings reported here provide a strong support for this conclusion: (*a*) the ability of NSC-13502 to block HIF-1 DNA binding of endogenous nuclear proteins from hypoxic treated cells, but only when the compound was added directly to the binding reaction, consistent with the notion that NSC-13502 binds DNA and not HIF-1α and/or HIF-1β proteins, and (*b*) the ability of NSC-13502 to block HIF-1 DNA binding to the HRE of VEGF promoter detected by ChIP. More importantly, NSC-13502 under the same experimental conditions did not block the binding of c-Fos to the AP-1 site of the cyclin D1 promoter or the binding of p65 to the NF-κB site of the ICAM-1 promoter. We believe that these results provide strong evidence that NSC-13502 interferes with HIF-1 DNA binding in a sequence-specific fashion. It is, however, conceivable that the sequence specificity of NSC-13502 may not be restricted to HIF-1 but may include other transcription factors that have similar core-binding sites in their DNA consensus sequences. One example is the Myc/Max family of transcription factors, whose DNA-binding site (E-box, 5′-CACGTG-3′; ref. 46) encompasses the core sequence 5′-CGTG-3′, which is also present in the HIF-1 consensus sequence and is part of the sequence specificity of NSC-13502. Indeed, we found that NSC-13502 completely inhibited DNA-binding activities to an oligonucleotide containing the E-box sequence detected in nuclear extracts harvested from untreated MCF-7 cells. Although this feature might detract from the target specificity of this small molecule, it may also become a desirable element for increased therapeutic activity.

Consistent with data from the primary high-throughput screen, NSC-13502 inhibited hypoxic induction of luciferase in U251-HRE cells and VEGF mRNA expression in U251 cells. Ultimately, the feasibility of using small molecules to interfere in a sequence-specific fashion with binding of transcription factors to DNA will be determined by their ability to modulate gene expression in a predictable fashion and by their activity in preclinical studies where validation of the inhibition of the target and its downstream genes will become essential. Global analysis of gene expression using oligonucleotide arrays containing sequences representing ~11,000 human genes showed that a relatively small number of genes are changed by addition of NSC-13502 under either normoxic or hypoxic conditions. In fact, addition of NSC-13502 to U251 cells cultured under normoxic conditions only down-regulated or up-regulated 276 and 186 genes, respectively, by ≥2-fold. Interestingly, a synthetic pyrrole-imidazole polyamide that inhibits in a sequence-specific fashion HIF binding to the HRE of the VEGF promoter (44) caused 264

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and 73 genes to be down-regulated and up-regulated by 2-fold, respectively, at a concentration of 1 μmol/L.

NSC-13502 was brought into clinical trials by the NCI 20 years ago based on its antitumor activity against two i.p. implanted murine tumors, the B16 melanoma and the P388 leukemia (47). NSC-13502, at 1.2 to 1.5 mg/m^2 once weekly for 4 weeks or once every 3 to 4 weeks (47–50), has been extensively tested in phase I-II clinical trials. Nausea, vomiting, reversible liver enzyme abnormalities, and allergic reactions were the most common toxicities encountered. However, minimal or no antitumor activity was found in phase II clinical trials. It remains to be established if, based on evidence presented in this article, alternative schedules of administration of NSC-13502 aimed at inhibiting HIF-1 activity might have a better therapeutic potential. Further experiments will determine whether this is a feasible approach to modulate HIF-1–dependent gene expression *in vivo* and to explore its potential therapeutic applications.

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