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Identification of Small Molecule Inhibitors of Hypoxia-inducible Factor 1 Transcriptional Activation Pathway[1]

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ABSTRACT

Hypoxia-inducible factor 1 (HIF-1) is a master regulator of the transcriptional response to oxygen deprivation. HIF-1 has been implicated in the regulation of genes involved in angiogenesis [*e.g.*, vascular endothelial growth factor (VEGF) and inducible nitric oxide synthase] and anaerobic metabolism (*e.g.*, glycolytic enzymes). HIF-1 is essential for angiogenesis and is associated with tumor progression. In addition, overexpression of HIF-1α has been demonstrated in many common human cancers. Therefore, HIF-1 is an attractive molecular target for development of novel cancer therapeutics. We have developed a cell-based high-throughput screen for the identification of small molecule inhibitors of the HIF-1 pathway. We have genetically engineered U251 human glioma cells to stably express a recombinant vector in which the luciferase reporter gene is under control of three copies of a canonical hypoxia-responsive element (U251-HRE). U251-HRE cells consistently expressed luciferase in a hypoxia- and HIF-1-dependent fashion. We now report the results of a pilot screen of the National Cancer Institute "Diversity Set," a collection of approximately 2000 compounds selected to represent the greater chemical diversity of the National Cancer Institute chemical repository. We found four compounds that specifically inhibited HIF-1-dependent induction of luciferase but not luciferase expression driven by a constitutive promoter. In addition, these compounds inhibited hypoxic induction of VEGF mRNA and protein expression in U251 cells. Interestingly, three compounds are closely related camptothecin analogues and topoisomerase (Topo)-I inhibitors. We show that concomitant with HIF-1 and VEGF inhibition, the activity of the Topo-I inhibitors tested is associated with induction of cyclooxygenase 2 mRNA expression. The luciferase-based high-throughput screen is a feasible tool for the identification of small molecule inhibitors of HIF-1 transcriptional activation. In addition, our results suggest that altered Topo-I function may be associated with repression of HIF-1-dependent induction of gene expression.

INTRODUCTION

Hypoxia, a decrease in O_2 levels, triggers adaptive responses in solid tumors that include induction of angiogenesis and a switch to anaerobic metabolism (1).

HIF-1[4] is a basic helix-loop-helix Per-Arnt-Sim transcription factor composed of two subunits, HIF-1α and HIF-1β (2). Two other homologues of the α subunit have been cloned (HIF-2α or EPAS-1 and HIF-3α), but there appears to be little redundancy in the hypoxic response. HIF-1β, also known as aryl hydrocarbon receptor nuclear translocator, is constitutively present in normoxic cells. In contrast, HIF-1α levels are primarily dependent on the intracellular oxygen concentration (3). Under nonhypoxic conditions, HIF-1α protein is rapidly and continuously degraded by ubiquitination and proteosomal degradation. Degradation of HIF-1α is dependent on binding with Von Hippel-Lindau and hydroxylation of Pro-564 via an enzymatic process that requires O_2 and iron (4, 5). However, under hypoxic conditions, HIF-1α protein accumulates and translocates to the nucleus, where it forms an active complex with HIF-1β and activates transcription of target genes by binding to the DNA consensus sequence 5′-RCGTG-3′. Besides physiological hypoxia, genetic abnormalities frequently detected in human cancers, including loss of function mutations (*i.e.*, Von Hippel-Lindau, p53, and PTEN), are associated with induction of HIF-1α activity and expression of HIF-1-inducible genes including but not limited to VEGF production (6–9).

HIF-1α plays a critical role in embryonic development. Indeed, genetic deletion of the HIF-1α subunit in mouse embryos resulted in developmental arrest and vascular abnormalities with embryonic lethality by embryon day 11 (10–12). HIF-1 is also essential for angiogenesis and tumor progression "*in vivo,*" as indicated by experiments in which tumor xenografts of HIF-1β-deficient hepatoma cells (13), HIF-1α-deficient H-ras-transformed cell lines (14), or embryonic stem cells from HIF-1$\alpha^{-/-}$ mice (11) showed decreased growth rate and vascularization relative to wild-type cells. More importantly, overexpression of HIF-1α protein has been demonstrated in many common human cancers (15) including prostate and breast cancer, in which HIF-1α levels were associated with increased vascularity and tumor progression (16). Finally, disruption of HIF-1α transcriptional activity, using a peptide that interferes with the interaction between HIF-1α and the coactivator p300/CREB-binding protein, has shown therapeutic activity in xenograft models of colon carcinoma and breast cancer (17), providing "proof of principle" that HIF-1 is a promising molecular target for development of cancer therapeutics.

We have developed a cell-based HTS to identify small molecule inhibitors of the HIF-1 pathway, which may have antiangiogenic and anticancer activities. U251 human glioma cells were genetically engineered to stably express a recombinant vector in which the luciferase reporter gene is under control of three copies of a canonical HRE. We now report the results of a pilot HTS of the NCI "Diversity Set," a collection of approximately 2000 compounds generated to maximally represent the three-dimensional chemical diversity in the whole NCI library (18). We identified four compounds that specifically inhibited luciferase expression driven by a HIF-1-inducible promoter but not by a constitutively active promoter. In addition, these compounds also inhibited in a dose-dependent fashion hypoxic induction of VEGF mRNA and protein expression in U251 cells. Interestingly, three compounds are closely related CPT analogues and Topo-I inhibitors (NSC-609699, NSC-606985, and NSC-639174).

In conclusion, we have developed a molecular targeted HTS that coherently identifies small molecule inhibitors of HIF-1 transcriptional activity and VEGF expression. Screening of larger chemical

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[4] The abbreviations used are: HIF, hypoxia-inducible factor; HRE, hypoxia-responsive element; VEGF, vascular endothelial growth factor; COX-2, cyclooxygenase 2; GLUT-3, glucose transporter type 3; HTS, high-throughput screen; PTEN, phosphatase and tensin homologue; DFX, desferrioxamine; SRB, sulforhodamine B; SI, specificity index; Topo, topoisomerase; CPT, camptothecin; NCI, National Cancer Institute; ActD, actinomycin D; TK, thymidine kinase; RT-PCR, reverse transcription-PCR; NF-κB, nuclear factor κB.

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libraries may lead to the identification of active compounds that could find interesting applications in anticancer treatment.

MATERIALS AND METHODS

Cell Lines and Reagents. We routinely maintained U251 human glioma cells in RPMI 1640 (Whittacker Bioproducts, Walkersville, MD) supplemented with 5% heat-inactivated fetal bovine serum (Whittacker Bioproducts), penicillin (50 IU/ml), streptomycin (50 μg/ml) and 2 mM glutamine (all purchased from Invitrogen-Life Technologies, Inc., Carlsbad, CA). Cells were maintained at 37°C in a humidified incubator containing 21% O_2, 5% CO_2 in air (referred to as normoxic conditions). Hypoxia treatment was performed by placing cells in a modular incubator chamber (Billupus-Rothemberg Inc., Del Mar, CA) and then flushing with a mixture of 1% O_2, 5% CO_2, and 94% nitrogen for 20 min. The chamber was then placed at 37°C. DFX was purchased from Sigma (St. Louis, MO), and ActD was purchased from Calbiochem (La Jolla, CA). Drugs from the NCI Training and Diversity Set (described elsewhere[5]) were initially dissolved in DMSO (Sigma) and diluted into complete medium for assay.

Plasmids. We generated pGL2-TK promoter plasmid by replacing the SV40 promoter of pGL2 promoter (Promega) with the herpes simplex virus TK promoter fragment (from −105 to +51).

The pGL2-TK-HRE plasmid was generated by subcloning three copies of the HRE (5′-GTGACTACGTGCTGCCTAG-3′) from the inducible nitric oxide synthase promoter into the pGL2-TK promoter vector (19). Plasmids were sequenced at the Molecular Technology Laboratory, Science Applications International Corporation-Frederick, Inc.

pGL3-control (Promega) contains the firefly luciferase coding sequence under control of the SV40 promoter and enhancer sequences.

Plasmid p7, containing the VEGF 5′-flanking sequence (−1005 to +306) upstream of the luciferase reporter gene, and p11WT, encompassing the HIF-1-binding site of VEGF promoter at position –985 and –939, were generous gifts from Dr. Gregg Semenza (20).

Stable Transfection and Engineered Cell Lines. DNA plasmids were prepared using a commercially available kit (Endofree Maxi-Prep; Qiagen, Inc., Valencia, CA). Transfections were performed using Effectene Transfection Reagents (Qiagen, Inc.) according to the manufacturer's instructions. Stably transfected cells (U251-HRE, U251-TK, U251-pGL3, U251-p7, and U251-p11) were generated by cotransfection of the specific reporter plasmid (pGL2-TK-HRE, pGL2-TK promoter, pGL3-control, VEGF-p7, or VEGF-p11WT, respectively) with an expression vector carrying the neomycin resistance gene for selection in mammalian cells (ratio, 100:1). Twenty-four h after transfection, reagents were removed, and cells were allowed to recover for 24 h before the addition of selection medium containing the antibiotic G418 at 500 μg/ml (Invitrogen-Life Technologies, Inc.). Stably transfected cells were seeded at a concentration of 1×10^4 cells/well in 96-well optiplates the day before treatment and routinely treated for 16–24 h.

Luciferase reporter assays were performed in 96-well optiplates (Packard Instrument, Inc., Meriden, CT) using Bright Glo luciferase assay reagents (Promega, Inc., Madison, WI).

Nuclear Extract Preparation and Immunoblotting (Western Blot). Cells were collected and washed twice with ice-cold Dulbecco's PBS 1× (PBS) and pelleted by centrifugation at 1,200 rpm. The cell pellet was subsequently washed once in a hypotonic buffer [10 mM Tris-HCl (pH 7.5), 1.5 mM $MgCl_2$, 10 mM KCl, 2 mM DTT, 1 mM Pefabloc, 2 mM sodium vanadate, 4 μg/ml pepstatin, 4 μg/ml leupeptin, and 4 μg/ml aprotinin], resuspended in the same buffer, and incubated for 10 min on ice. The cell suspension was homogenized with 18–20 strokes in a glass Dounce homogenizer. The nuclear pellet was obtained after centrifugation at $1,000 \times g$ at 4°C for 10 min and resuspended in a hypertonic buffer [20 mM Tris-HCl (pH 7.5), 1.5 mM $MgCl_2$, 0.42 M KCl, 20% glycerol, 2 mM DTT, 1 mM Pefabloc, 2 mM sodium vanadate, 4 μg/ml pepstatin, 4 μg/ml leupeptin, and 4 μg/ml aprotinin] to obtain nuclear extracts. The nuclear suspension was rotated at 4°C for 30 min, and nuclear debris were pelleted by centrifugation at $15,000 \times g$ for 30 min at 4°C.

Typically, 50 μg of protein were separated on an 8% Tris-glycine gel (Invitrogen Corp., Carlsbad, CA) and electroblotted on an Immobilon-P membrane (Invitrogen Corp.). Membranes were blocked for 1 h at room temperature with blocking buffer A (1× PBS, 0.1% Tween 20, and 4% BSA) to detect HIF-1α and blocked with blocking buffer B (1× PBS, 0.1% Tween 20, and 5% nonfat dry milk) to detect HIF-1β before incubation with primary antibodies (1 h at room temperature) in dilution buffer (1× PBS, 0.1% Tween 20, and 0.4% BSA). Monoclonal anti-HIF-1α (clone H1α67) and monoclonal anti-HIF-1β (clone H1B234) antibodies were purchased from Novus Biologicals (Littleton, CO). HIF-1α was detected using a 1:1000 dilution of the specific antibody, whereas HIF-1β antibody was diluted 1:1500. After washing three times in washing buffer (1× PBS and 0.1% Tween 20), membranes were incubated for 30 min at room temperature with a peroxidase-conjugated sheep antimouse antibody (diluted 1:20000 in dilution buffer) for both HIF-1α and HIF-1β (Amersham Pharmacia Biotech, Inc., Piscataway, NJ). Membranes were then washed three times in washing buffer, and chemiluminescence detection was performed using an enhanced chemiluminescence kit according to the manufacturer's protocol (Amersham Pharmacia Biotech, Inc.).

Electrophoretic Mobility Shift Assay. Nuclear extracts, prepared as described previously, were dialyzed against one change of dialysis buffer [25 mM Tris-HCl (pH 7.5), 0.2 mM EDTA, 0.1 M KCl, 20% glycerol, 1 mM DTT, 0.2 mM Pefabloc, and 0.5 mM sodium vanadate]. The double-stranded oligonucleotide AB.2 (5′-GTGCTACGTGCTGCCTAG-3′) encompassing the HIF-1 binding site was labeled with [^{32}P]dCTP using the Klenow enzyme (Invitrogen-Life Technologies, Inc.). To perform the binding reaction, 5 μg of nuclear protein were incubated on ice in binding buffer [25 mM Tris-HCl (pH 7.5), 0.2 mM EDTA, 0.1 M KCl, 20% glycerol, and 0.4 μg of denatured calf thymus DNA] in the presence or absence of antibodies for HIF-1α for 30 min. Probe (1×10^4 cpm) was added, and the samples were incubated for an additional 20 min on ice. Samples were subjected to a 5% nondenaturing polyacrylamide gel, and electrophoresis was performed at 180 V in 0.3× Tris-borate EDTA at 4°C. Gel was allowed to dry using HydroTech gel drying system (Bio-Rad Laboratories, Hercules, CA) before being autoradiographed using Kodak XAR-5 film and intensifying screens at −80°C.

HTS Assay. U251-HRE cells were inoculated into 384 well white flat-bottomed plates (Costar catalogue number 3704) at 3000 cells/well with a Beckman Biomek 2000 Laboratory Automation Workstation in a volume of 25 μl and incubated for 24 h at 37°C, 5% CO_2, and ambient O_2. Experimental agents at the appropriate concentrations were added in a volume of 25 μl using the Biomek 2000, and after a 20-h incubation in a hypoxia chamber (Billups Rothenberg, MIC 101) at 37°C, 5% CO_2, and 1% O_2, the plates were removed and incubated at room temperature, 5% CO_2, and ambient O_2 for 1.5 h. Forty μl of Bright Glo luciferase reagent (Promega catalogue number E26500) were added with the Biomek 2000, and after 3 min, luminescence was measured using a Tecan Ultra Multifunction Plate Reader in luminescence mode. Appropriate control cells were treated identically, except that they were treated at 37°C, 5% CO_2, and ambient O_2. Compound toxicity was assayed using the SRB assay as described previously in detail (21).

Real-time PCR. Total RNA from U251 cells was obtained using RNA Mini Kit (Qiagen, Inc.). One μg of total RNA was used to perform RT-PCR using RT-PCR kit (PE Biosystems, Foster City, CA). The conditions used for RT-PCR were as follows: 10 min at 25°C, 30 min at 48°C, and 5 min at 95°C. To measure human VEGF, COX-2, GLUT-3, and aldolase expression, real-time PCR was performed using an ABI-Prism 7700 Sequence Detector (Applied Biosystems, Foster City, CA). Typically 5 ng of reverse-transcribed cDNA per sample were used to perform real-time PCR in triplicate samples. Real-time PCR cycles started with 2 min at 50°C, 10 min at 95°C, and then 40 cycles of 15 s at 95°C and 1 min at 60°C. Primers and specific probes were obtained from Applied Biosystems. The following primers and probes were used: human VEGF forward, 5′-TACCTCCACCATGCCAAGTG-3′; human VEGF reverse, 5′-ATGATTCTGCCCTCCTCCTTC-3′; probe, 5′-FAM-TCCCAGGCTGCACCCATGGC-TAMRA-3′; human COX-2 forward, 5′-GAATCATTCACCAGGCAAATTG-3′; human COX-2 reverse, 5′-TCTGTACTGCGGGTGGAACA-3′; probe, 5′-FAM-TGGCAGGGTTGCTGGTGGTAGGA-TAMRA-3′; human GLUT-3 forward, 5′-CGTGGCAGGACTTTTGAGGAT-3′; human GLUT-3 reverse, 5′-AGCAGGCTCGATGCTGTTCAT-3′; human aldolase forward, 5′-GCGCTGTGTGCTGAAAATCAG-3′; and human aldolase reverse, 5′-CCACAATAGGCACAATGCCATT-3′. Detection of 18S rRNA, used as internal control, was preformed using premixed reagents from Applied Biosystems. Detection of VEGF, COX-2, and 18S rRNA was performed using TaqMan Universal PCR Master Mix (Applied

[5] http://dtp.nci.nih.gov.

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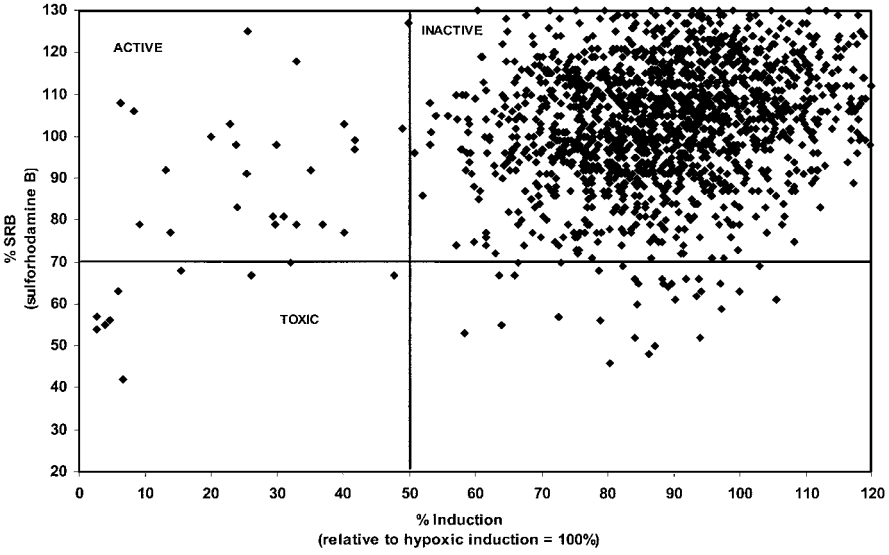

Fig. 1. Results of the diversity set HTS. U251-HRE cells were inoculated into 384-well white flat-bottomed plates at 3000 cells/well in a volume of 25 μl and incubated for 24 h at 37°C, 5% CO_2, and ambient O_2. Experimental agents (1 μM) were added in a volume of 25 μl. After a 20-h incubation in the hypoxia chamber at 37°C, 5% CO_2, and 1% O_2, the plates were removed and incubated at room temperature and ambient O_2 for 1.5 h before luminescence was measured. Compound toxicity was assayed using the SRB assay. Data from the HTS are plotted as percentage induction of luciferase expression (relative to hypoxia alone, equal to 100%) on the *X* axis and percentage toxicity (relative to untreated cells, equal to 100%) on the *Y* axis for each individual compound.

Biosystems), whereas GLUT-3 and aldolase detection was performed using Sybr Green PCR Master Mix (Applied Biosystems).

Relative quantitation values were expressed as follows: $2^{(\Delta Ctr-\Delta Ctt)}$, where *C* is the value measured in each well**,** *Ct* is the mean of the replicate wells run for each sample, ΔCt is the difference between the mean *Ct* values of the samples in the target wells and those of the endogenous control for the same wells (18S values), $\Delta Ctr-\Delta Ctt$ represents the difference between ΔCt of the reference sample (medium) and ΔCt of the tested samples (treatments). Values are expressed as fold increases relative to the reference sample (medium).

ELISA. Supernatants were collected from U251 cells after 24 h of incubation under the indicated conditions. Total levels of VEGF protein were measured at the Lymphokine Testing Laboratory, Science Applications International Corporation-Frederick, Inc., NCI (Frederick, MD) using human VEGF DuoSet (R&D Systems, Minneapolis, MN).

RESULTS

Development of a HTS Targeting HIF-1 Transcriptional Activity. To engineer human cancer cells to express the luciferase reporter gene under control of a HIF-1-inducible promoter, we cotransfected pGL2-TK-HRE containing three copies of a canonical HRE with a vector containing the neomycin gene for selection in mammalian cells. Surprisingly, we found that hypoxic inducibility of luciferase expression was greatly diminished or totally abrogated in many cell lines upon stable transfection of pGL2-TK-HRE (MCF-7, PC-3, H4-60, OVCAR-3, DU145, A549, MDA-MB-435, and HCT-116) compared with the levels of induction observed in transient transfection experiments (data not shown). In contrast, hypoxic induction of luciferase was largely preserved in U251-HRE cells. In fact, U251-HRE cells expressed low but detectable levels of luciferase in normoxic conditions but expressed significantly higher levels (15 ± 3.1-fold above normoxic control in 10 independent experiments) in cells cultured under hypoxic conditions. DFX also increased luciferase expression to levels comparable with those induced by hypoxia (20 ± 2.8-fold above normoxic control in 10 independent experiments; data not shown).

To identify small molecule inhibitors of HIF-1 transcriptional activity, we developed a HTS using U251-HRE cells. Initial characterization of the assay was achieved by testing the NCI Training Set, a collection of approximately 200 compounds representative of the major mechanistic classes of standard anticancer drugs as well as a variety of specific inhibitors of enzymes or signaling pathways. Results obtained from two independent experiments showed that there was a very high correlation coefficient (0.938; $R^2 = 0.88$), suggesting that results of the HTS were consistent and reproducible. In addition, a statistical parameter developed to evaluate and validate HTS assays (Z-factor) was routinely assessed for quality control purposes (22). Z statistic calculated for the 20 plates of the Diversity Set measuring the "screening window" between normoxic and hypoxic cells was 0.632 ± 0.076, indicative of a statistically significant separation. Based on the consistent performance of U251-HRE cells in HTS, we scaled up to screen a library of approximately 2000 compounds (NCI Diversity Set), which represents a collection of diverse chemical structures available at NCI.

U251-HRE cells were cultured in normoxic or hypoxic conditions for 24 h in the presence or absence of drugs from the NCI Diversity Set at a concentration of 1 μM. Toxicity and inhibition of cell growth were routinely assessed in a parallel SRB assay. In a primary screen, 35 compounds inhibited hypoxic induction of luciferase expression in U251-HRE cells by a factor of ≥50%; 26 of them had little or no toxicity or cell growth inhibition (≤30%) in the SRB assay (Fig. 1). Additional experiments were then aimed at confirming the results of the primary screen and identifying the EC_{50} (the dose at which there was 50% inhibition of hypoxic induction of luciferase) of the 26 positive nontoxic compounds.

Parallel experiments were performed to exclude compounds that inhibited luciferase expression in a nonspecific and/or HIF-1-independent fashion. To this purpose, we developed an engineered cell line (U251-pGL3) in which the luciferase reporter gene is under the control of a constitutively active promoter. U251-pGL3 cells expressed high basal levels of luciferase in normoxic conditions and slightly lower levels in hypoxic conditions (data not shown). We defined the EC_{50} of the 26 compounds identified in the primary screen using U251-HRE and U251-pGL3 cell lines and calculated a SI, obtained by dividing the EC_{50} in U251-pGL3 by the EC_{50} in U251-HRE, which provides an indication of relative specificity toward inhibition of HIF-1-dependent transcription. As shown in Fig. 2, seven compounds had a SI ≥ 10 (chosen as arbitrary threshold), indicating that they had minimal or no activity on the constitutive expression of luciferase in the U251-pGL3 control cell line. Similar results were obtained using a control cell line engineered to express luciferase under control of the minimal TK promoter (U251-TK; data not shown).

In conclusion, 7 compounds out of approximately 2000 compounds

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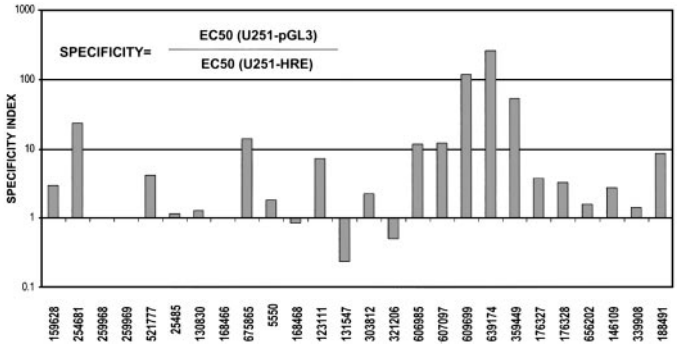

Fig. 2. Summary of the SI of 26 compounds identified in the primary HIF-1 HTS. U251-HRE cells were treated as described in Fig. 1 in the presence of an increasing concentration of the indicated compounds under normoxic or hypoxic conditions. U251-pGL3 cells were treated identically, except that they were treated at 37°C, 5% CO_2, and ambient O_2. EC_{50}s were calculated for each compound, and a SI was determined by dividing EC_{50} U251-pGL3/EC_{50} U251-HRE. Compound toxicity was assayed using the SRB assay.

that compose the NCI Diversity Set preferentially inhibit luciferase expression driven by HIF-1-inducible promoters.

The HIF-1-targeted HTS Coherently Identifies Molecules That Inhibit HIF-1 Transcriptional Activity and VEGF Expression. To test whether the compounds identified in the HTS inhibited the endogenous activity of HIF-1, we incubated U251 cells under normoxic or hypoxic conditions for 8 h in the presence or absence of drugs (0.5 μM) and measured VEGF mRNA expression by real-time PCR. We found that hypoxia induced up to 7-fold higher levels of VEGF mRNA expression in U251 cells relative to normoxic control (Fig. 3). Moreover, hypoxia induced up to ~4-fold higher levels of VEGF protein (2450 pg/ml) above the constitutive levels expressed under normoxic conditions (570 pg/ml). Four of the seven compounds identified in the HTS inhibited hypoxic induction of VEGF mRNA expression by more than 50% relative to hypoxia alone, one showed minimal but not statistically significant inhibition (NSC-254681), and two were inactive. Consistent with these results, hypoxic induction of VEGF protein was inhibited in a similar fashion (data not shown).

Among the four compounds that inhibited hypoxic induction of VEGF mRNA expression in U251 cells, three are CPT analogues and Topo-I inhibitors (NSC-609699, NSC-606985, and NSC-639174) (23), and the remaining one is a quinocarmycin analogue, DX-52-1 (NSC-607097; Ref. 24; Fig. 4). The chemical structure of the remaining three compounds that do not have inhibitory effects on VEGF expression is also shown (Fig. 4).

In conclusion, our results suggest that the HTS coherently identifies molecules that interfere with HIF-1 transcriptional activity and with the hypoxic induction of VEGF expression.

NSC-609699 Specifically Inhibits Hypoxic Induction of VEGF Expression. Of the three CPT analogues identified in the HTS, NSC-609699 (topotecan) is the best characterized for its activities both *in vitro* and in the clinical setting. Therefore, we decided to further investigate the effects of NSC-609699 on hypoxic induction of luciferase expression in U251-HRE cells. As shown in Fig. 5, NSC-609699 inhibited hypoxic induction of luciferase by 79%, 45%, and 27% at 500, 50, and 5 nM, respectively, with an EC_{50} of 71.3 nM. In contrast, NSC-609699 only inhibited constitutive expression of luciferase by 14% in the U251-pGL3 control cell line at the highest concentration used, without reaching an EC_{50}. Parallel SRB analysis showed that NSC-609699 decreased cell viability by only 15% at the higher concentration used, under both normoxic and hypoxic conditions. NSC-609699 also inhibited DFX-dependent induction of luciferase expression in U251-HRE cells in a dose-dependent manner with an EC_{50} of 181 nM (data not shown). The other two CPT analogues identified in the HTS, NSC-606985 and NSC-639174, also inhibited hypoxic induction of luciferase expression in U251-HRE cells with an EC_{50} of 32.7 and 477 nM, respectively (data not shown).

Inhibition of Topo-I activity has been associated with modulation of gene expression and in particular with activation of the ubiquitous transcription factor NF-κB, which plays an important role in the regulation of proinflammatory and antiapoptotic genes. To establish whether NSC-609699 had a differential effect on hypoxic induction of gene expression under our experimental conditions, U251 cells were incubated under normoxic or hypoxic conditions for 8 h in the presence or absence of an increasing concentration of NSC-609699 (from 1 nM to 1 μM), and expression of VEGF mRNA and COX-2 mRNA was evaluated. COX-2 is a hypoxia-inducible proinflammatory gene whose expression is controlled, at least in part, by NF-κB. As shown above, hypoxia induced 6-fold higher levels of VEGF mRNA expression relative to normoxic conditions. NSC-609699 had no effect on accumulation of VEGF mRNA under normoxia at the concentrations tested. In contrast, NSC-609699 had a dramatic inhibitory effect on hypoxic induction of VEGF mRNA expression, with 45% inhibition at 10 nM and 78% inhibition at 1 μM (Fig. 6*A*). Additional experiments also indicated that NSC-609699 inhibits hypoxic induction of other HIF-1-dependent genes in U251 cells, including expression of GLUT-3 and aldolase mRNA by 53% and 60%, respectively (data not shown). Interestingly, we found that NSC-609699 caused a dose-dependent induction of COX-2 mRNA expression under normoxic conditions. In particular, NSC-609699 induced a 2-fold accumulation of COX-2 mRNA at 1 nM and induced up to a 21-fold increase at 1 μM. In addition, hypoxia induced COX-2 mRNA expression (6-fold above baseline), and NSC-609699 had an additive effect when combined with hypoxia, with an 18-fold increase above normoxic levels at 100 nM and up to a 25-fold increase at 1 μM (Fig. 6*B*).

These results demonstrate that NSC-609699 has a differential effect on hypoxic induction of gene expression. Under our experimental conditions, inhibition of Topo-I activity seems to be associated with down-regulation of HIF-1-dependent transcription and activation of NF-κB-dependent responses.

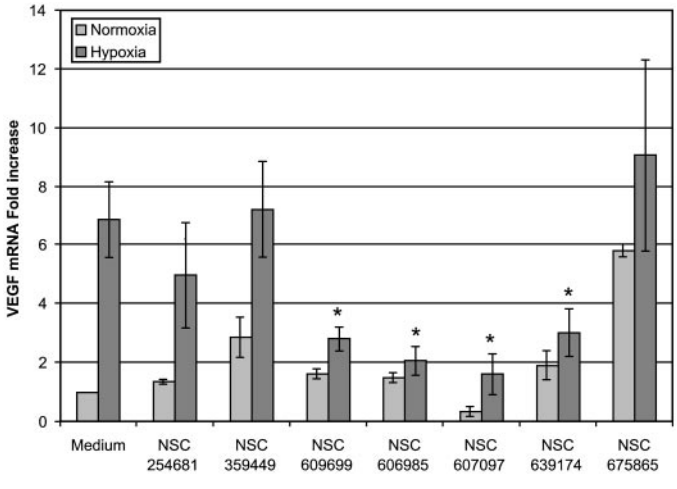

Fig. 3. Inhibition of hypoxic induction of VEGF mRNA expression in U251 cells. U251 cells (2×10^5 cells/well) were seeded in 6-well plates and incubated for 8 h under normoxic or hypoxic conditions, in the presence or absence of the indicated compounds (0.5 μM). Total RNA was harvested and tested for VEGF mRNA expression by real-time PCR, as described in "Materials and Methods." Results are expressed as fold increase relative to VEGF mRNA levels under normoxic conditions (equal to 1) in the absence of drugs. 18S rRNA was tested in parallel as internal control for input RNA. Results are the average ± SE of three independent experiments. Statistical analysis was performed using ANOVA (two-factor with replication) test ($P < 0.05$).

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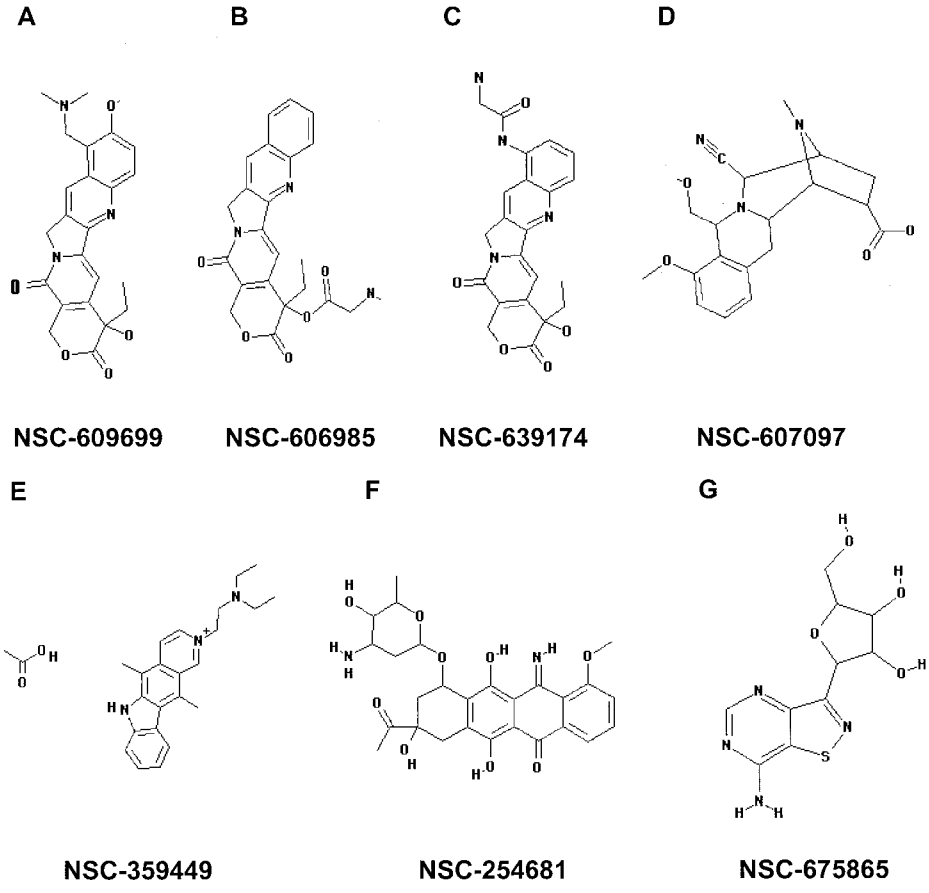

Fig. 4. Chemical structures of positive hits. *A*, NSC-609699, topotecan. *B*, NSC-606985, camptothecin, 20-ester(*S*). *C*, NSC-639174, 9-glycineamido-20(*S*)-camptothecin HCl. *D*, NSC-607097, DX-52-1, quinocarmycin analogue. *E*, NSC-359449, 2-(5,11-dimethyl-6H-2λ^5-pyrido[4,3-b]carbazol-2-yl)-*N*,*N*-diethylethanamine acetate. *F*, NSC-254681, 3-acetyl-3,5,12-trihydroxy-11-imino-10-methoxy-6-oxo-1,2,3,4,6,11-hexahydro-1-naphthacenyl 3-amino-2,3,6-trideoxy-hexopyranoside. *G*, NSC-675865, 1-(7-aminoisothiazolo[4,5-d]pyrimidin-3-yl)-1,4-anhydropentitol.

Inhibition of VEGF Expression by NSC-609699 Is Transcriptional. To assess whether NSC-609699 inhibited VEGF expression at the transcriptional level, we genetically engineered U251 cells to stably express VEGF-p7 containing the luciferase reporter gene under control of a 1.0-kb fragment of the VEGF promoter or VEGF-p11WT, in which the luciferase gene is under the control of a 50-bp oligonucleotide encompassing the active HIF-1 binding site of the VEGF promoter. U251-p7 cells expressed higher levels of luciferase when cultured under hypoxic conditions (2 ± 0.29-fold above normoxic control in five independent experiments) or in the presence of DFX (2.5 ± 0.06-fold above normoxic control; data not shown). U251-p11 cells also expressed higher levels of luciferase either under hypoxic conditions (3.6 ± 0.49-fold above normoxic control) or in the presence of DFX (4.3 ± 0.29-fold; data not shown). U251-p7 and U251-p11 cells were cultured under normoxic or hypoxic conditions in the presence or absence of increasing concentrations of compound NSC-609699. As shown in Fig. 7*A*, NSC-609699 inhibited hypoxic induction of luciferase expression in U251-p7 by 69.7%, 52.3%, and 26.8% at 500, 50, and 5 nM, respectively, with an EC_{50} of 51.2 nM. In addition, NSC-609699 inhibited hypoxia induction of luciferase expression in U251-p11 by 71%, 50.1%, and 30.2% at 500, 50, and 5 nM, respectively, with an EC_{50} of 61.6 nM. NSC-609699 also inhibited DFX-dependent induction of luciferase expression in U251-p7 and U251–p11 cells in a dose-dependent manner with an EC_{50} of 199 and 223 nM, respectively (data not shown). In contrast, experiments performed in the presence or absence of ActD (5 μg/ml) indicated that induction of COX-2 mRNA expression by NSC-609699 is due, at least in part, to transcriptional activation. In fact, NSC-609699 caused a 7-fold increase above basal levels of COX-2 mRNA expression that was completely abrogated by the addition of ActD (Fig. 7*B*).

These data are consistent with inhibition of HIF-1 transcriptional activity and demonstrate that NSC-609699 mediates transcriptional repression of VEGF mRNA expression.

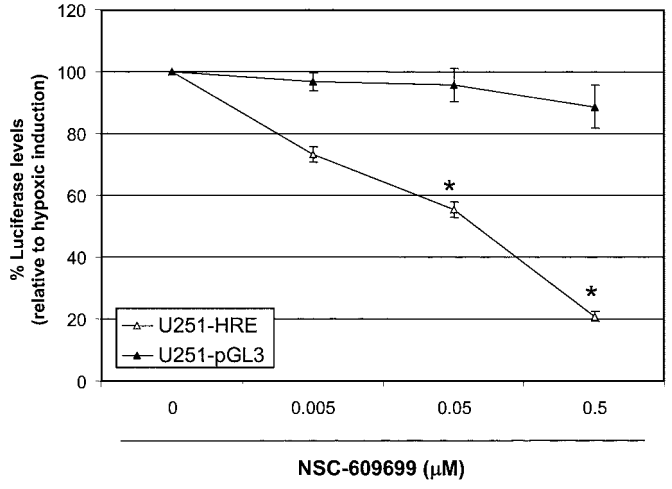

Fig. 5. NSC-609699 specifically inhibits hypoxic induction of luciferase expression in U251-HRE, but not in U251-pGL3. U251-HRE and U251-pGL3 cells (1×10^4 cells/well) were seeded in 96-well optiplates and incubated under normoxic or hypoxic conditions in the presence or absence of the indicated concentration (μM) of NSC-609699. SRB assay was performed on parallel plates to monitor toxicity. Cells were treated for 24 h and then tested for cell viability and luciferase expression. Results are expressed as percentage of luciferase levels (normalized to SRB data) induced under hypoxic conditions (equal to 100%). Data are presented as the average ± SE of six independent experiments. Statistical analysis was performed using ANOVA (two-factor with replication) test ($P < 0.01$). U251-HRE cells, △; U251-pGL3 cells, ▲.

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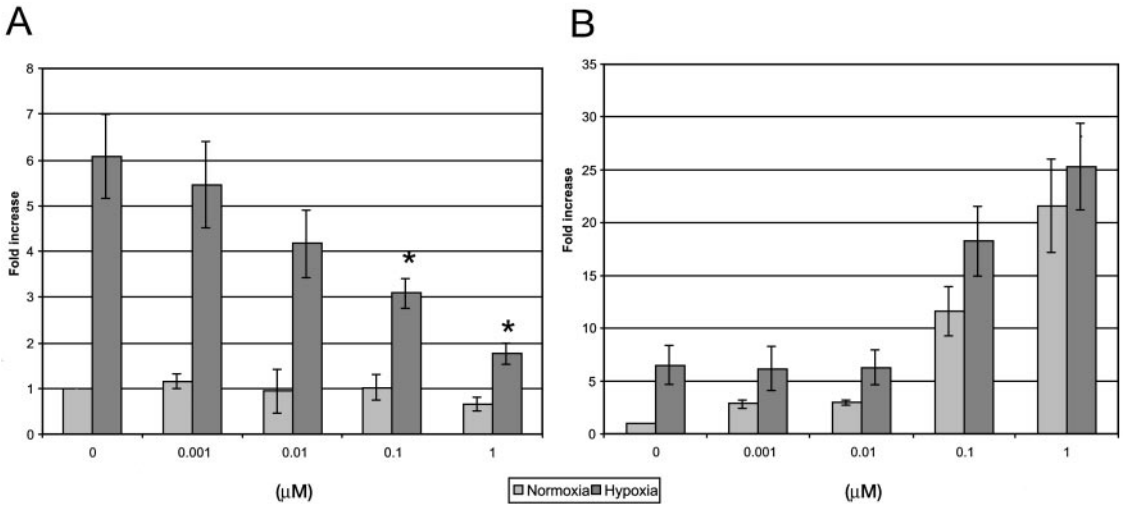

Fig. 6. NSC-609699 differentially regulates VEGF mRNA and COX-2 mRNA expression in U251 cells. U251 cells (2×10^5 cells/well) were seeded in 6-well plates and incubated under normoxic or hypoxic conditions for 8 h, in the presence or absence of increasing concentrations (μM) of NSC-609699 as indicated. Total RNA was harvested and processed for expression of VEGF and COX-2 mRNA by real-time PCR as described. 18S rRNA was tested in parallel to control for input RNA. Results are expressed as fold increase relative to levels of mRNA under normoxic conditions in the absence of drug (equal to 1). Data are presented as the average ± SE of four independent experiments. Statistical analysis was performed using ANOVA (two-factor with replication) test ($P < 0.01$).

NSC-609699 Specifically Inhibits Hypoxic Induction of HIF-1α Protein and DNA Binding Activity. To further investigate the mechanism by which NSC-609699 inhibited HIF-1-dependent transcriptional activation, U251 cells were incubated under normoxic or hypoxic conditions in the presence or absence of NSC-609699 (0.1 μM), and protein levels of HIF-1α and HIF-1β were measured by Western blot. As shown in Fig. 8*A*, we found that U251 cells express low but detectable basal levels of HIF-1α protein at 6 h and slightly higher levels at 24 h, which were substantially increased (up to 6-fold) by incubation under hypoxic conditions at both time points. NSC-609699 decreased the basal levels of HIF-1α protein under normoxic conditions and caused significant inhibition of hypoxic-dependent accumulation of HIF-1α protein by approximately 70% at 6 h and complete abrogation at 24 h. In contrast, HIF-1β was constitutively expressed under nonhypoxic conditions, and its levels were not changed by incubation under hypoxia or by addition of NSC-609699.

In parallel experiments, we examined the effects of NSC-609699 on hypoxic induction of DNA binding activity to an oligonucleotide encompassing a canonical HIF-1 binding site. Nuclear extracts from U251 cells cultured under normoxic conditions have a constitutive binding activity that appears as a doublet (Fig. 8*B, lanes 1* and *5*, band C) and a complex of slower mobility (band I) more appreciable at 24 h. Hypoxia increased the appearance of the inducible complex at 6 and 24 h (band I, Fig. 8*B, Lanes 3* and *7*). Both binding activities were competed for by an excess of unlabeled specific probe, and the inducible complex was entirely supershifted by the addition of a specific anti-HIF-1α antibody, but not by the addition of an isotype-matched irrelevant antibody (data not shown). NSC-609699 did not affect the constitutive binding activity but significantly decreased at 6 h (Fig. 8*B, Lane 4*) and completely abrogated at 24 h (Fig. 8*B, Lane 8*) the appearance of the hypoxia-inducible DNA binding complex. Direct addition of up to 1 μM NSC-609699 in the binding reaction did not affect the hypoxia-inducible complex (data not shown), suggesting that NSC-609699 does not directly interfere with the formation of protein-DNA complex.

These results indicate that NSC-609699-dependent inhibition of HIF-1 transcriptional activation is due, at least in part, to a down-regulation of hypoxia-dependent accumulation of HIF-1α protein.

DISCUSSION

HIF-1 is an attractive molecular target for development of novel cancer therapeutics. To identify small molecule inhibitors of HIF-1 transcriptional activation, we have developed a HTS using engineered cell lines that express the luciferase reporter gene in a HIF-1-inducible fashion. Our results using U251-HRE cells to screen the NCI Diversity Set indicate a coherent identification of molecules that inhibit HIF-1 activity and VEGF expression.

HIF-1 activates transcription of target genes by binding to a HRE that contains the HIF-1 core DNA binding site 5′-RCGTG-3′. We have previously demonstrated that a 19-bp element from the inducible nitric oxide synthase promoter containing a canonical HIF-1 binding site (5′-TACGTG-3′) mediates hypoxic and DFX inducibility upon transient transfection in mammalian cells (19, 25). Previous experiments also indicated that a 3-bp mutation of the HIF-1 binding site completely abrogated hypoxic and HIF-1 inducibility of a reporter gene and DNA binding activity (19). We have exploited these characteristics of the HRE to generate engineered human cancer cell lines expressing the luciferase reporter gene in a HIF-1-inducible fashion. Most cell lines lost hypoxic inducibility upon stable transfection of the luciferase reporter vector, despite efforts to identify high-inducible clones by limiting dilution. In contrast, U251-HRE cells express high levels of luciferase consistently and reproducibly upon incubation under hypoxic conditions. Experiments shown in this study were performed with "bulk" transfected U251-HRE cells, which eliminates concerns regarding clonal variability of expression and drug sensitivity. Interestingly, we have tested U251-HRE cells routinely for up to 6 months in culture, and we have observed that hypoxic inducibility was largely preserved, although basal levels of luciferase expression were variable and decreased with higher number of passages in culture (data not shown).

U251 cells have a complex genetic background that may have profound influences on the HIF-1 pathway. In particular, U251 have a mutation of the PTEN tumor suppressor gene (26) and consequent activation of the phosphatidylinositol 3′-kinase/Akt pathway, which is involved in cell survival and regulation of hypoxic responses (9, 27). Recent evidence has indicated that PTEN loss of function increases responsiveness to hypoxic induction of HIF-1 transcriptional activity

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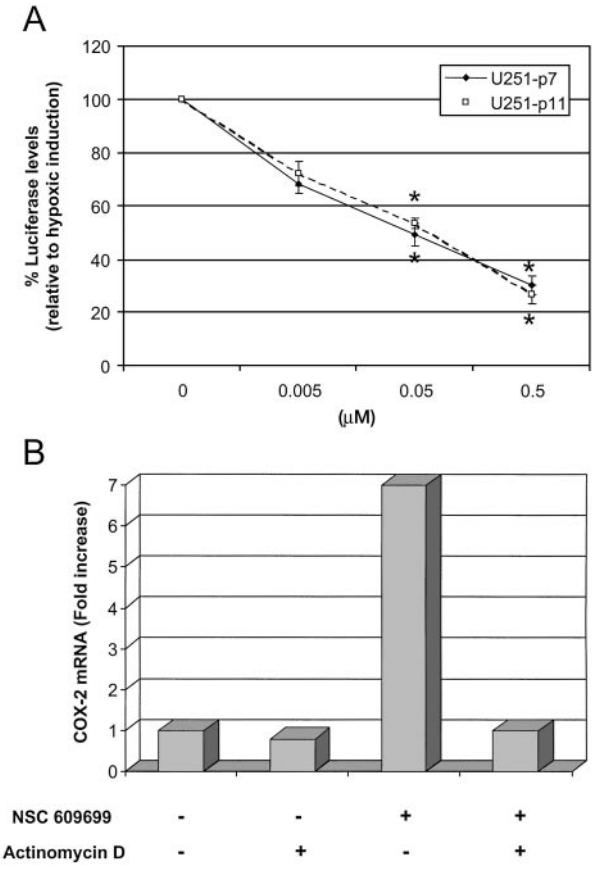

Fig. 7. NSC-609699 specifically inhibits VEGF mRNA expression at the transcriptional level. *A*, U251-p7 and U251-p11 cells (1×10^4 cells/well) were seeded in 96-well optiplates and incubated under normoxic or hypoxic conditions in the presence or absence of the indicated concentration (μM) of NSC-609699. SRB assay was performed on parallel plates to monitor toxicity. Cells were treated for 24 h and then tested for cell viability and luciferase expression. Results are expressed as percentage of luciferase levels (normalized to SRB data) induced under hypoxic conditions (equal to 100%). Data are presented as the average ± SE of four independent experiments. Statistical analysis was performed using ANOVA (two-factor with replication) test ($P < 0.01$). U251-p7 cells, ♦; U251-p11 cells, □. *B*, U251 cells (2×10^5 cells/well) were seeded in 6-well plates and incubated under normoxic conditions for 6 h, in the presence or absence of NSC-609699 (0.1 μM) and ActD (5 μg/ml). Total RNA was harvested and processed for expression of COX-2 mRNA by real-time PCR as described. 18S rRNA was tested in parallel to control for input RNA. Results are expressed as fold increase relative to levels of mRNA present under normoxic conditions in the absence of drugs (equal to 1). Data presented are representative of three independent experiment performed.

with consequent increased expression of genes involved in angiogenesis and survival (7). We found that U251 cells do express low basal levels of HIF-1α protein and DNA binding activity under normoxic conditions. However, U251 cells are very responsive to hypoxic stimulation with induction of HIF-1α protein, DNA binding activity, and luciferase reporter gene expression. Whether the particular genetic make-up makes U251-HRE cells such a suitable model for investigating HIF-1 transcriptional activation remains to be fully determined. The wide window of hypoxic inducibility makes U251-HRE a unique model for drug development applications targeting HIF-1 transcriptional activity. Conversely, these unique features of U251-HRE cells raise the possibility that our screening assay is biased toward identification of agents that act specifically in glioma cells. However, PTEN mutations are common in human cancers, and identification of compounds that act specifically in tumor cells harboring genetic abnormalities would enhance the therapeutic window of HIF-1 inhibitors for clinical development.

The NCI Diversity Set is a collection of approximately 2000 compounds representative of chemical diversity from the larger Developmental Therapeutics Program repository and contains alkylating agents, antimitotic agents, RNA and DNA antimetabolites, Topo-I and Topo-II inhibitors, and DNA-binding and -interacting agents. Because inhibition of transcription is the end point of the HIF-1-targeted HTS, we were particularly concerned of a potential bias of our screen toward preferential identification of compounds that interact with DNA. To minimize this possibility, we have developed control cell lines that express luciferase in a constitutive, HIF-1-independent fashion. In addition, we have selected compounds of interest based on lack of toxicity (as assessed by SRB assay) and high SI (relative ratio of specificity toward inhibition of HIF-1-dependent transcription). Although the compounds identified in the primary screen are known to be DNA-interacting agents when used at high concentrations (28–31), the NCI Diversity Set contains many other DNA-interacting agents that have been rejected from this screen. Therefore, the performance of DNA-interacting agents in our cell-based screen cannot be generalized, and the built-in system of controls is stringent enough to discriminate compounds that act in a nonspecific and/or HIF-1-independent fashion.

We have basically identified two different structures that interfere with HIF-1 transcriptional activity. NSC-607097, known as DX-52-1, is a more stable analogue of quinocarmycin (32). Quinocarmycin and DX-52-1 were evaluated at the NCI in the early 1990s using a disease-oriented drug screen system and were first identified as having melanoma specificity. In particular, seven of eight melanoma lines were more sensitive than average to DX-52-1. In addition, DX-52-1 demonstrated *in vivo* antitumor activity against melanoma using staged s.c. implanted human xenograft models (24). Supported by these data, DX-52-1 was developed to Phase I clinical trials, but its use was discontinued because of unexpected and unpredictable toxicities. Whether inhibition of HIF-1 transcriptional activation played a role in the performance of the drug in the clinical trial is unknown. We have found that NSC-607097 inhibits HIF-1-dependent luciferase expression with an EC_{50} of 170 nM (data not shown) but with a relatively low SI, which raises the possibility that DX-52-1 has

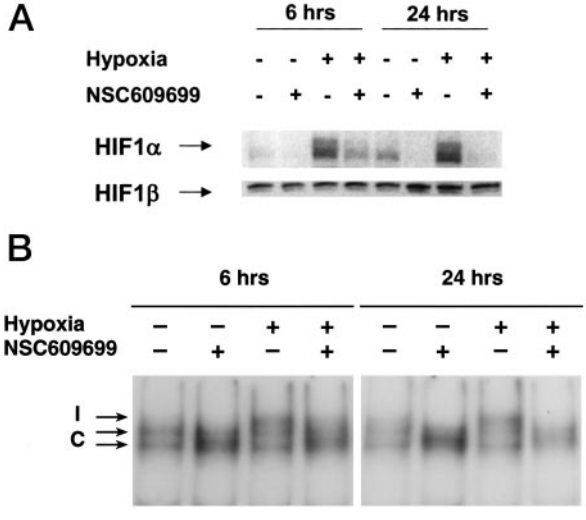

Fig. 8. NSC-609699 specifically inhibits HIF-1α protein accumulation and DNA binding activity. *A*, U251 cells were incubated under normoxic or hypoxic conditions in the presence or absence of NSC-609699 (0.1 μM) for 6 and 24 h, and then nuclear extracts were prepared as described in "Materials and Methods." Fifty μg of protein were separated on an 8% Tris-glycine gel. Specific monoclonal antibodies for detection of HIF-1α and HIF-1β were used as described. Results shown are from one representative experiment of four performed. *B*, U251 cells were cultured under normoxic or hypoxic conditions as described in *A*) for 6 and 24 h, and then nuclear extracts were prepared as described. Electrophoretic mobility shift assay was performed with radiolabeled AB.2 probe as described. Binding activities are labeled as follows: *C*, constitutive; and *I*, inducible. Results shown are from one experiment of at least three performed.

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nonspecific effects at higher concentrations and may have a narrow therapeutic window. Interestingly, results of a parallel HTS using U937 cells engineered to express luciferase under the control of a CAAT/enhancer binding protein α-responsive promoter indicated that NSC-607097 (100 nM) induced luciferase expression to levels comparable to retinoic acid, a known activator of this pathway.[6] These data suggest that NSC-607097 has a differential effect on transcription factors, which may have distinct consequences on gene expression.

The second class of compounds identified in the HTS is represented by drugs that inhibit Topo-I activity, topotecan and two CPT analogues. CPTs belong to a class of chemotherapeutic agents that stabilize the complex formed between Topo-I and DNA (33). In the presence of CPTs, Topo-I remains covalently linked to one strand of the DNA and thus leaves the DNA with a protein-linked DNA single-stranded break, which triggers cytotoxic lesions in metabolizing DNA (30, 31). Besides DNA damage, inhibition of Topo-I has been associated with activation of the NF-κB pathway (23, 34) and inhibition of angiogenesis (35, 36). We have found that inhibition of Topo-I activity by topotecan is associated, under our experimental conditions, with HIF-1 transcriptional repression and down-regulation of HIF-1-dependent gene expression, with an EC_{50} in the low nanomolar range. Inhibition of HIF-1 transcriptional activity and VEGF mRNA expression by NSC-609699 was also observed in other human cancer cell lines including MCF-7, P388, CEM, and DU145 (data not shown). Consistent with these results, topotecan also inhibited hypoxic induction of luciferase expression driven by a 1.0-kb fragment of the VEGF promoter that encompasses the HIF-1 binding site, suggesting that topotecan inhibits VEGF transcription. Concomitant with inhibition of VEGF mRNA expression, topotecan independently induced COX-2 mRNA expression and had an additive effect with hypoxia, demonstrating that it mediates a distinct pattern of gene expression. Recently, therapeutic strategies aimed at blocking NF-κB activation associated with Topo-I inhibition have been proposed to decrease NF-κB-dependent antiapoptotic effects (37). Our results raise the possibility that inhibition of COX-2 activity, which has been associated with angiogenic and tumor-promoting effects (38), may become an independent target of therapeutic strategies aimed at improving the clinical efficacy of CPT.

Topo-I inhibitors may have a profound effect on gene expression. It has recently been shown that mRNA levels for particular genes may rise or fall in response to CPT treatment (39). The response of individual genes to CPT analogues may result directly from Topo-I inhibition (such as the immobilization of Topo-I complex on the DNA and sequential DNA damage) or may arise through secondary mechanisms [such as inhibition of direct protein-protein interaction between Topo-1 and other transcription factors (40) or recruitment of transcription factors through the immobilization of Topo-1 on specific DNA sequences (41)]. We found that inhibition of HIF-1 activity occurs in both transiently and stably transfected cells, suggesting that this activity is independent of DNA conformation (data not shown). More importantly, our results indicate that NSC-609699 selectively inhibits accumulation of HIF-1α protein and consequently the appearance of DNA binding activity, which correlates nicely with inhibition of VEGF mRNA expression. Whether a direct inhibition of Topo-I activity is essential for inhibition of HIF-1α protein accumulation and VEGF expression is currently under investigation in our laboratory. It is conceivable, however, that inhibition of Topo-I activity may be associated with more profound effects on transcription and may not be restricted to HIF-1. In this respect, it may become difficult to discriminate the contribution of HIF-1 inhibition in the clinical setting, and this feature of topotecan or CPT analogues may not be fully exploitable in the clinical setting.

In conclusion, screening of larger chemical libraries using cancer cell lines engineered with HIF-1-inducible promoters may lead to identification of HIF-1 inhibitors that could be developed for clinical application. Accordingly, the DTP has undertaken a HIF-1-targeted HTS campaign of a 140,000-compound library available at the NCI. Although the potential contribution of HIF-1 inhibitors to cancer therapeutics is largely speculative at this time, accumulating information on the involvement of HIF-1 in cancer progression makes HIF-1 an attractive target for development of novel therapeutic strategies.

[6] D. Scudiero, M. Selby, T. Silvers, J. Laudeman, S. Clopper, J. Grams-Fowler, D. Tenen, H. Radomska, E. Sausville, and R. Shoemaker, Development of a cell-based high throughput screen for inducers of C-EBPα, manuscript in preparation.

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