Identification of Novel Small Molecule Inhibitors of Hypoxia-Inducible Factor-1 That Differentially Block Hypoxia-Inducible Factor-1 Activity and Hypoxia-Inducible Factor-1α Induction in Response to Hypoxic Stress and Growth Factors

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Abstract

Hypoxia-inducible factor-1 (HIF-1) is a transcriptional complex that is activated in response to hypoxia and growth factors. HIF-1 plays a central role in tumor progression, invasion, and metastasis. Overexpression of the HIF-1α subunit has been observed in many human cancers and is associated with a poor prognostic outcome with conventional treatments. Targeting HIF-1 using novel small molecule inhibitors is, therefore, an attractive strategy for therapeutic development. We have generated U2OS human osteosarcoma cells stably expressing a luciferase reporter construct under the control of a hypoxia response element (U2OS-HRE-luc). The U2OS-HRE-luc cells were robustly and reproducibly sensitive to hypoxic stress in a HIF-1-dependent manner. We developed an automated U2OS-HRE-luc cell-based assay that was used in a high-throughput screen to identify compounds that inhibited HIF-1 activity induced by treatment with the hypoxia mimetic, deferoxamine mesylate. We performed a pilot screen of the National Cancer Institute Diversity Set of 2,000 compounds. We identified eight hit compounds, six of these were also identified by Rapisarda et al. in an independent hypoxia screen. However, there were two novel hit compounds, NSC-134754 and NSC-643735, that did not significantly inhibit constitutive luciferase activity in U2OS cells (U2OS-luc). We showed that both NSC-134754 and NSC-643735 significantly inhibited HIF-1 activity and HIF-1α protein induced by deferoxamine mesylate. Interestingly, NSC-134754 but not NSC-643735 inhibited HIF-1 activity and HIF-1α protein induced by hypoxia and significantly inhibited Glut-1 expression. Finally, we showed that both NSC-134754 and NSC-643735 inhibited HIF-1α protein induced by insulin-like growth factor-1. Our cell-based assay approach has successfully identified novel compounds that differentially target hypoxia and/or growth factor-mediated induction of HIF-1α. (Cancer Res 2005; 65(11): 4918-28)

Introduction

Solid tumors characteristically contain areas of hypoxia (low oxygen tension), which is a powerful stimulus for the expression of genes involved in proliferation, glycolysis, and angiogenesis. Adaptation of tumor cells to a hypoxic environment results in an aggressive and metastatic cancer phenotype that is associated with resistance to radiation therapy, chemotherapy, and a poor treatment outcome (1–5).

The transcription factor hypoxia-inducible factor-1 (HIF-1) is central to the regulation of a growing number of hypoxia-activated genes and consists of HIF-1α and HIF-1β subunits. The mRNAs of HIF-1α and HIF-1β, also known as the aryl hydrocarbon receptor nuclear translocator (ARNT), are constitutively expressed in cells. However, HIF-1α protein expression is tightly regulated by changes in cellular oxygen and growth factors. Both subunits contain a basic-helix-loop-helix and PER-ARNT-SIM (PAS) domain important for heterodimerization and DNA binding. Two other α-subunits have been identified in addition to HIF-1α and are called HIF-2α and HIF-3α. Interestingly, HIF-3α has been shown to exist as a number of splice variants (6) and is thought to negatively regulate the HIF pathway (7, 8).

HIF-1α subunit is primarily regulated at the level of protein stability. In normoxia, HIF-1α is rapidly degraded via targeted ubiquitination and subsequent degradation by the proteasome. This negative regulation is mediated by direct binding of the von Hippel-Lindau (VHL) tumor suppressor protein, a component of an E3 ubiquitin ligase, and is dependent on prolyl hydroxylation of HIF-1α at residues 402 and 564 (9, 10). In response to physiological hypoxia, HIF-1α becomes rapidly stabilized and is localized to the nucleus, where it binds to HIF-1β to form the HIF-1 complex. HIF-1 specifically binds to a short DNA sequence, 5′-ACGTG-3′, as known as the hypoxia responsive element (HRE) within target genes. Upon binding, HIF-1 recruits the coactivator CAMP-responsive element binding protein (CREB)-binding protein/p300 and various other proteins to activate transcription (11, 12).

HIF-1 plays a central role in tumor progression and angiogenesis in vivo. Oncogenic activation (e.g., Ha-ras, myc, or src) or loss of tumor suppressor function (e.g., p53, PTEN, or VHL) is associated with HIF-1-mediated tumor progression (13, 14). In addition, exposure to a variety of growth factors [insulin, insulin-like growth factor-1 (IGF-1), IGF-2, and angiogenins I and II] has also been shown to increase HIF-1 activity in normoxic and hypoxic conditions. HIF-1α is overexpressed in many human cancers (15). The resistance of hypoxic cells to killing and the aggressiveness of highly hypoxic tumors are, in part, due to the overexpression of HIF-1α. The recent finding showing that radiation itself activates HIF-1 adds to the therapeutic challenge of a vascular hypoxic tumor (2, 16). Elegant studies have shown that targeting the interaction between HIF-1α and the p300/CREB-binding protein...
significantly reduces tumor growth in vivo and support HIF-1 as a potential therapeutic target (17, 18). Consequently, an increase in the development of strategies to target HIF-1 activity has intensified in recent years.

A variety of small molecule inhibitors of HIF-1 activity have been shown to have antitumor and antiangiogenic activity in vivo. These include the microtubule depolymerizing agent 2-methoxyestradiol (19); inhibitors of the redox protein, thioredoxin-1 (20, 21); YC-1, an agent developed for circulatory disorders (22); PX-478, a small molecule inhibitor that targets both constitutive and hypoxia-induced HIF-1α levels (23); topotecan, a Topo-I inhibitor identified in a cell-based high-throughput hypoxia screen (24, 25); the Hsp90 inhibitors, geldanamycin and its analogue 17-allylamino,17-demethoxygeldanamycin (26); and the small molecule inhibitor, chemotin, which blocks the interaction between HIF-1α and p300/CREB (18).

In this study, we have developed a robust cell-based assay that we have used in a high-throughput screen to identify small molecule inhibitors of the HIF pathway. We performed a pilot screen of the National Cancer Institute (NCI) Diversity Set of 2,000 compounds, enabling us to compare our hits with a previous hypoxia screen (24). In addition to identifying six hit compounds, which were previously described (24), we discovered two novel hit compounds, NSC-134754 and NSC-643735, which we evaluated further. We show here that NSC-134754 and NSC-643735 differentially block HIF-1 activity and HIF-1α expression in response to hypoxic stress. Importantly, we show that NCS-134754 blocks both the hypoxia and growth factor–mediated pathways regulating HIF-1α expression.

Materials and Methods

Plasmid constructs and antibodies. A full-length human HIF-1α expression construct (pCMV-HA-HIFα) was provided by Dr. Andrew Kung (Dana-Farber Cancer Institute, Boston, MA). The pGL-HRE luciferase reporter construct contains a triple repeat of the iNOS HRE binding sequence and was kindly provided by Dr. Giovanni Melillo (NCI, Bethesda, MD). The pGL3-basic and control vectors were obtained from Promega (Southampton, United Kingdom). The HIF-1α–specific monoclonal antibody was obtained from BD Biosciences (Oxford, United Kingdom). The β-actin–specific monoclonal antibody was obtained from Abcam (Cambridge, United Kingdom). The Glut-1 antibody polyclonal antibody was obtained from Alpha Diagnostic International (Wiltshire, United Kingdom).

Cell culture. Stable transfection was used to generate the U2OS-HRE-luc, U2OS basic, and U2OS luc control cells using the constructs pGL-HRE, pGL3-basic, and pGL3-control, respectively. The pGL3-HRE construct has three tandem copies of the iNOS HRE fused to the luciferase reporter gene. The pGL3-control vector also contains the luciferase reporter gene and expression is driven by SV40 promoter and enhancer sequences resulting in constitutive luciferase activity. The pGL3-basic vector contains the luciferase reporter gene only. pSV2γ-hygromycin was cotransfected and selection was carried out using hygromycin (Boehringer Mannheim, Berkshire, United Kingdom). Stable clones were isolated and expanded for further evaluation. All cell lines were maintained in DMEM (Life Technologies, Paisley, United Kingdom) containing 10% FCS and cultured at 37°C in 5% CO2. Transient transfections were carried out using the calcium phosphate precipitation method. Cells were harvested either in passive lysis buffer (Promega, Southampton, United Kingdom) or in 2× sample buffer [125 mmol/L Tris (pH 6.8), 4% SDS, 0.01% bromophenol-blue, 10% [–mercaptopethanol, 10% glycerol] to assess luciferase activity or protein expression respectively. For assessment of protein expression samples were separated by SDS-PAGE and Western blot analysis was done as previously described (27).

HIF-1α induction. Cells were exposed to hypoxic conditions (1% O2, 5% CO2, and 94% N2) in a LEEC dual gas incubator at 37°C.expression samples were separated by SDS-PAGE and Western blot analysis was done as previously described (27).

HIF-1α induction. Cells were exposed to hypoxic conditions (1% O2, 5% CO2, and 94% N2) in a LEEC dual gas incubator at 37°C. HIF-1 was induced in normoxia using the hypoxia mimetic agent deferoxamine mesylate (DFX, Sigma, Poole, United Kingdom) at a concentration of 500 μmol/L. A stock of DFX (500 mmol/L) was freshly prepared in sterile H2O immediately before each experiment. For experiments using the phosphorosidase 3 kinase inhibitor, cells were treated with 10 or 80 μmol/L. I1294002 (Calbiochem, Nottingham, United Kingdom) for 15 minutes before exposure to hypoxia (1% O2) or treatment with DFX (500 μmol/L) for up to 16 hours. For experiments using NSC-134754, NSC-643735, and NSC-607097, cells were treated with 20 μmol/L of each compound (unless otherwise indicated) for 30 minutes before exposure to hypoxia (1% O2) or treatment with DFX (500 μmol/L) for up to 16 hours. For treatment with IGF-1, HCT116 cells were starved for 36 to 48 hours then stimulated with 50 ng/mL recombinant IGF-1 (Sigma) for 6 to 8 hours as described previously (27). Compounds were added to the cells 30 minutes before IGF-1 stimulation.

High-throughput screening assay. U2OS-HRE-luc cells were grown in DMEM complete medium with 10% fetal bovine serum and their use in a high-throughput screen initially evaluated using 96-well plates. The assay was subsequently satisfactorily transferred to a 384-well plate format for compound screening. U2OS-HRE-luc cells were plated into 384-well plates at 4,000 cells/well. The following day, compounds were added 30 minutes before DFX treatment (500 μmol/L). The final concentration of compounds was 18.18 μmol/L. Total activity controls included DMSO (final concentration 0.2%) plus DFX (n = 32), and the positive control was I1294002 (final concentration 80 μmol/L, n = 18). Other controls were DMSO only (n = 16) and DFX only (n = 18). The plates were incubated overnight at 37°C in a humidified incubator. Luciferase reporter activity was measured using the ReporterLight Plus assay reagents (Cambrex, Berkshire, United Kingdom) according to the manufacturer’s instructions and after 10 minutes the plates were read on a Topcount plate reader (Perkin-Elmer Life Sciences, Buckinghamshire, United Kingdom). The mean Z’ factor, calculated using the DMSO plus DFX versus the DMSO only controls, was 0.52 ± 0.09. It is worth noting that a thorough comparison of the SteadyGlo (Promega) and Lumitech ReportaLight Plus luciferase reagents was made. Both reagents were comparable in terms of their sensitivity and duration of effectiveness. However, the latter was used in the high-throughput screen because it was more economical.

Hits were identified as those compounds that inhibited the readout (DFX plus DMSO) by greater than three standard deviations of the mean of the compounds on each plate. These compounds were selected and their activity confirmed (n = 4) in the same assay. To exclude the possibility that the inhibition was due to loss of cells, compounds were assayed at the same concentration in an ATP viability assay (Cambrex, Berkshire, United Kingdom) according to the manufacturer’s instructions. In addition, the effect of the hits on luciferase activity was determined using the ViaLight reagent in a cell-free assay using ATP (1 μmol/L) as a substrate. All compounds were prepared and stored in DMSO (2%). To control for any effect that might have been due to the DMSO, cells were either untreated or DMSO was added to untreated cells at the same final concentration and volume as used for compound treated cells.

 Luciferase reporter assays. HIF-1 activity was determined in the U2OS-HRE-luc cells, U2OS-basic-luc cells, or U2OS-luc control cells in six-well format. Cells were washed twice with ice-cold PBS and lysed with 200 μL of Passive Lysis buffer (Promega) and incubated at room temperature for 10 minutes. Lysates were centrifuged and supernatants were stored at −80°C until assayed. Luciferase assays were done by pipetting 20 μL of cell lysate into each well of a 96-well plate and analyzed immediately after addition of luciferase reagent (Promega) in a luminometer (Dynex Technologies, Worthing, United Kingdom).

Sulforhodamine-B assay. Analysis of cell growth inhibition was done by sulforhodamine-B assay. Cells were plated into clear flat-bottomed 96-well plates and left to attach overnight. They were treated with 5 μL/well of serially diluted compounds in quadruplicate and incubated for 72 hours. The cells were cultured in a final volume of 200 μL. At the end of the incubation period, the medium was removed and the cells were fixed immediately with ice-cold 10% trichloroacetic acid (VWR International, Poole, United Kingdom) for 30 minutes. The cells were then washed five times with H2O and allowed to dry before staining with 0.4% (w/v) sulforhodamine B (Sigma) dissolved in 1% acetic acid for 10 minutes.
Unbound dye was removed by five washes with 1% acetic acid, and protein-bound dye was extracted with 10 mmol/L unbuffered Tris base for 5 minutes for determination of absorbance in a microplate reader. The absorbance value was read at a wavelength of 540 to 570 nm.

**Results**

Effect of hypoxic stress on luciferase activity and HIF-1α induction in U2OS-HRE-luc cells. We have generated a human osteosarcoma cell line (U2OS) stably expressing a triple tandem repeat of a HRE fused to a luciferase reporter gene (U2OS-HRE-luc). To assess whether the U2OS-HRE-luc cells could be developed into a high-throughput cell-based assay to identify HIF-1 inhibitors, we initially characterized the responsiveness of the U2OS-HRE-luc cells to hypoxic stress. To do this, U2OS-HRE-luc cells were exposed to either hypoxia (1% O2) or treated with the hypoxia mimetic agent, DFX at 500 μmol/L over a time course of 24 hours. In response to hypoxia, the highest increase in luciferase activity was achieved at 16 hours of incubation (Fig. 1A). Interestingly, there was a decrease in HIF-1α protein levels at 24 hours of hypoxic treatment. We have observed this decrease in many other cell lines including MRC-5, MCF-7, U87MG, RPE-1, and HCT116 (data not shown) and the response is consistent with observations described elsewhere (28). This was in contrast to the response of DFX-treated U2OS-HRE-luc cells in which HIF-1α protein levels continued to increase with increasing luciferase activity being exhibited over a 24-hour period (Fig. 1B). The results suggest that increases in luciferase activity in the U2OS-HRE-luc cells were due to HIF-1 activation as a consequence of HIF-1α induction. To support this, the cells were transiently transfected with a HIF-1α expression construct or control vector. As seen in Fig. 1C, the presence of exogenous HIF-1α

![Figure 1](image-url)

**Figure 1.** Effect of hypoxic stress on luciferase activity and HIF-1α induction in U2OS-HRE-luc, U2OS-basic, and U2OS-luc control cells. U2OS-HRE-luc cells were exposed to normoxia, hypoxia (1% O2, A) or (B) DFX (500 μmol/L) for the times indicated. Samples were assayed for luciferase activity. Graph shows luciferase reporter activity as a fold induction relative to normoxia (norm). Experiments were done in duplicate. C, U2OS-HRE-luc cells were transiently transfected with 10 μg HIF-1α or the vector control (−), expression constructs. Twenty-four hours after transfection, cells were harvested for luciferase assay and Western blot analysis. Graph shows luciferase reporter activity as a fold induction relative to normoxia. Experiments were done in duplicate. Western blot, analysis (bottom) shows HIF-1α protein expression. Actin was used as a load control. D, U2OS-HRE-luc cells or U2OS-basic-luc cells were exposed to normoxia (norm), hypoxia (Hyp, 1% O2) or DFX (500 μmol/L) for 8 hours. Graph shows relative light units (RLU). Experiments were done in duplicate. E, U2OS-luc control cells were exposed to normoxia (norm), hypoxia (Hyp, 1% O2) or DFX (500 μmol/L) for 8 hours. Graph shows relative light units. Experiments were done in duplicate.
led to an increase in luciferase activity compared with vector control, confirming that luciferase activity is HIF-1–dependent, consistent with our previous observations (26). In addition to generating the U2OS-HRE-luc cell line, U2OS cells stably expressing a basic luciferase construct, which did not contain any HRE sequences, were also generated (U2OS-basic). As expected, no significant increase in luciferase activity was observed in response to hypoxia or DFX treatment in the U2OS cells stably expressing the basic luciferase vector (Fig. 1E). To address whether hypoxia or DFX treatment itself affected luciferase activity, we also generated a U2OS cell line stably expressing a control luciferase reporter, which contains an SV40 site and enhancer sequences (U2OS-luc). Figure 1E suggests that these cells have constitutively high basal luciferase activity in normoxia as expected. Interestingly, exposure of the U2OS-luc cells to hypoxia (1% O2) resulted in a significant decrease in the luciferase activity, suggesting that hypoxia affected luciferase reporter gene expression. DFX treatment of these cells had no significant effect on luciferase activity (Fig. 1E).

**Development of the U2OS-HRE-luc cell-based assay.** We next wanted to assess the robustness of the hypoxia-mediated response in the U2OS-HRE-luc cells. A recent study using U251-HRE cells in a high-throughput hypoxia screen described a reoxygenation of cells for 1.5 hours immediately after hypoxia and before lysis (24). We were particularly interested in assessing both HIF-1 activity and HIF-1α induction in response to hypoxia and upon reoxygenation of the U2OS-HRE-luc cells. U2OS-HRE-luc cells were exposed to normoxia or hypoxia for 6 hours. Cells were either lysed immediately or reoxygenated by exposure to normoxia before lysis. Consistent with previous studies (29), HIF-1α protein levels were significantly reduced by at least 50% upon reoxygenation of the cells, presumably via rapid degradation by the proteasome (Fig. 2A). Consequently, luciferase reporter activity was also significantly reduced (Fig. 2B). This was observed even when cells were exposed to hypoxia for longer periods (for up to 16 hours; data not shown). Our data suggested that performing a high-throughput hypoxia screen as previously described (24) would not be feasible using the U2OS-HRE-luc cells, because we wanted to ensure that luciferase reporter activity was a direct measure of HIF-1α protein induction. We therefore used DFX as an alternative method for HIF-1α stabilization. By way of validating the U2OS-HRE-luc cells in an automated high-throughput cell-based assay in response to DFX, the effects of DFX versus normoxia were assessed in 96-well format. We have previously shown that the phosphoinositide 3-kinase inhibitor LY294002 completely blocks HIF-1α protein expression and HIF-1 activity in the U2OS-HRE-luc cells (27). We, therefore, assessed HIF-1 activity in response to DFX in the presence and absence of LY294002. Cells were untreated or treated with either DMSO or LY294002 30 minutes before DFX treatment and incubated for 16 hours. Luciferase activity was assessed and the coefficient of variation (%) is shown above each column. The mean Z′ factor for the screen was found to be satisfactory at 0.52 ± 0.09.

**High-throughput screen of 2,000 compounds.** Following the validation and automation of the U2OS-HRE-luc cell-based assay, a pilot screen of ~2,000 compounds from the NCI Diversity Set was done in 384-well format. This enabled us to make a direct comparison of our data with those obtained from an independent cell-based screen done in hypoxic conditions (24). Eight hit compounds were identified from the NCI Diversity Set of 2,000 compounds. This represented a hit rate of 0.4%. The criteria for a hit in the screen were defined as any compound that caused a reduction in DFX-induced luciferase activity by more than three standard deviations from the control, and did not result in more than 30% growth inhibition in a 24-hour viability assay nor interfere with the luciferase reagent alone to cause a change in readout by >50%. We identified six hit compounds (NSC-675865, NSC-607097, NSC-259968, NSC-259969, NCS-25485, and NCS-31547) that were also identified in an independent hypoxia screen (Table 1; ref. 24). Four of these compounds (NSC-259968, NSC-259969, NSC-25485, and NSC-31547) were excluded because of nonspecific DNA binding activity (Table 1). In addition, we identified two novel compounds, NSC-134754 and NSC-643735, which we evaluated further (Table 1). Interestingly, topotecan (NSC-609699), camptothecin 20-ester(S) (NSC-606985), and camptothecin analogue, 9-glycineamido-20-(S)-camptothecin (NSC-639174), which were described as hits by Rapisarda et al. (24) also inhibited luciferase activity in our screen by 43.4%, 79.8%, and 23.9%, respectively. However, these compounds did not satisfy our criteria for a hit and were, therefore, not evaluated further.
Furthermore, we found that NSC-359449 and NSC-254681 had minimal effects on luciferase activity and although Rapisarda et al. (24) described these compounds as hits, they did not inhibit vascular endothelial growth factor mRNA expression.

Nonspecific effects of NSC-134754, NSC-607097, and NSC-643735. Cell-based assays may potentially identify compounds that act on multiple pathways. Therefore, evaluation of the hits in a variety of secondary assays is required for assessment of their mode of action against the intended target. Our primary interest was to address whether the hit compounds inhibited HIF-1 activity and HIF-1α protein levels in response to hypoxia. In addition, the nonspecific effects of the compounds on reporter genes were investigated. Of the hit compounds identified, three were studied in secondary assays. Two of these, NSC-134754 and NSC-643735, were discovered as novel hits in our screen. NSC-134754 is a semisynthetic analogue of the natural alkaloid, emetine (30), and NSC-643735 is a structural analogue of actinomycin D aglycone (31). The third, NSC-607097, a quinocarmycin analogue (32), was also identified by Rapisarda et al. (24) and was included for comparison. The chemical structures of the three compounds are shown in Fig. 3. To rule out any nonspecific effects on luciferase activity, the compounds were initially assessed on U2OS-luc control cells, which were the U2OS cells stably expressing a constitutive luciferase reporter construct (Fig. 4). Our results indicated that both NSC-134754 and NSC-607097 inhibited the luciferase activity nonspecifically to some degree. To determine whether a compound was more inhibitory of luciferase activity increased by HIF-1 and, therefore, could be considered as a selective HIF-1 inhibitor, a ratio of the percentage inhibition of HIF-1 activity in the U2OS-HRE-luc cells over the percentage inhibition under the same treatment condition in the U2OS-luc control cells was calculated. A compound was considered to have a greater effect on inhibiting HIF-1 activity compared with a nonspecific effect if an arbitrary ratio of 2 or above was obtained. The experiments using U2OS-HRE-luc and U2OS-luc control cells were performed in parallel so that a direct comparison could be made. Analysis of the results showed that NSC-134754 inhibited hypoxia and DFX-induced activity over nonspecific activity by a ratio of >2. The same was observed for NSC-643735 following DFX treatment. However, NSC-607097 was borderline in terms of specificity as the ratio of inhibition in U2OS-HRE-luc cells versus U2OS-luc control cells was 1.4 in hypoxia

<table>
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<th>Hit compounds</th>
<th>%Inhibition primary screen</th>
<th>%Inhibition reconfirmation (n = 4)</th>
<th>%Growth inhibition at 24 h</th>
<th>%Inhibition luciferase reagent (24)</th>
<th>Compounds excluded from both screens</th>
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NOTE: Hit compounds identified by both screens (+). Two novel compounds (NSC-134754 and NSC-643735) were identified in our screen. NSC-259968, NSC-259969, NSC-25485, and NSC-131547 were excluded from both screens because of their nonspecific DNA binding activity (*). NSC-609699, NSC-606985, and NSC-639174 are camptothecin and its analogues (*). They were inhibitory in our screen, but they did not meet the criteria used for identification of a hit in our assay.

Table 1. Comparison of compounds identified in the screen done by Rapisarda et al. (24) with those identified in our high-throughput screen

![Figure 3. Chemical structures of NSC-134754, NSC-607097, and NSC-643735.](cancerres.aacrjournals.org)
although it was >2 for DFX. All three compounds were, therefore, found to have HIF-1-specific effects and were evaluated further.

**Effects of NSC-134754, NSC-607097, and NSC-643735 on HIF-1 activity and HIF-1α protein levels.** To determine whether the compounds decreased hypoxia-induced HIF-1 activity, 20 μmol/L of each compound was added to U2OS-HRE-luc cells 30 minutes before treatment with DFX (500 μmol/L) or exposure to hypoxia (1% O₂) for 16 hours. Graph shows luciferase reporter activity as relative light units. Each experiment was done in duplicate. Figure 4. Effect of NSC-134754, NSC-607097, and NSC-643735 on luciferase activity of U2OS-luc control cells. U2OS-luc control cells were treated with (A) 20 μmol/L NSC-134754 (lane A), NSC-607097 (lane B), NSC-643735 (lane C), or vehicle control (DMSO) 30 minutes before exposure to normoxia (norm) or DFX (500 μmol/L) for 16 hours. Graph shows luciferase reporter activity as relative light units. Each experiment was done in duplicate. (B) Western blot analysis shows HIF-1α protein expression. Actin was used as a load control. Graph shows luciferase reporter activity as relative light units. Experiments were done in duplicate.

Figure 4. Effect of NSC-134754, NSC-607097, and NSC-643735 on luciferase activity of U2OS-luc control cells. U2OS-luc control cells were treated with (A) 20 μmol/L NSC-134754 (lane A), NSC-607097 (lane B), NSC-643735 (lane C), or vehicle control (DMSO) 30 minutes before exposure to normoxia (norm) or DFX (500 μmol/L) for 16 hours. Graph shows luciferase reporter activity as relative light units. Each experiment was done in duplicate. (B) Western blot analysis shows HIF-1α protein expression. Actin was used as a load control. Graph shows luciferase reporter activity as relative light units. Experiments were done in duplicate.

**Discussion**

Before embarking on any drug discovery project, one of the fundamental questions to be answered is why a particular target is valid for therapeutic intervention. To satisfactorily answer this question, it is necessary to show that the target has a greater relevance in disease than in health. There are several lines of evidence to indicate that elevated levels of HIF-1α and its resultant
activity promotes tumor progression and the development of treatment resistance (33–35). In addition, it has also been shown that inhibiting HIF-1α can reduce tumor growth in vivo (17, 18, 36, 37). Accordingly, much interest has been generated in the search for HIF-1 inhibitors in recent years.

The aim of this study was to develop and implement a strategy to therapeutically target the HIF pathway. In the absence of any known direct enzymatic regulators of HIF that would be feasible to inhibit in a biochemical assay, we developed a cell-based assay for a high-throughput screen to identify small molecule inhibitors of HIF-1. Here, we describe the generation, characterization, and validation of the U2OS-HRE-luc cell-based assay and the outcome of a high-throughput pilot screen using this system. We also generated secondary cell-based assays (U2OS-basic-luc and U2OS-luc control cells) that were suitable for evaluating the hit compounds in initial deconvolution studies, with the aim of establishing their specificity for the HIF-1 pathway.

The validation and optimization of the U2OS-HRE-luc cell-based assay showed that this was a robust and reproducible system that could be used to identify HIF-1 inhibitors. A high-throughput pilot screen of the NCI Diversity Set of ~2,000 compounds was done. Importantly, we also did a viability assay in parallel with the high-throughput screen to enable us to establish the toxicity of the compounds in the U2OS-HRE-luc cell-based assay. Interestingly, a comparison of our hit compounds with those identified from a hypoxia screen done at the NCI recently (24) showed that many of the hit compounds were identical, despite a difference in cell line, the concentration at which the test compounds were screened (1 versus 18 μmol/L), and most notably a difference in the mode by which HIF-1 activity was induced in both screens. Our screen was performed using the hypoxia mimetic DFX. Rapisarda et al. (24) identified topotecan and several camptothecin analogues as primary hits. Indeed, we also found that topotecan and camptothecin inhibited HIF-1 activity in our own screen. However, they did not meet our
criteria for progression as a HIF-1–selective hit. Although the values were very close to be considered a hit by our criteria, they did not reproducibly cause a reduction in DFX-induced luciferase activity by more than three standard deviations from the control and, therefore, were not evaluated further. This does not mean, however, that the effects of these compounds are not interesting or therapeutically relevant. Topotecan is currently in use for treatment of ovarian cancer and non–small cell lung carcinoma (24, 38) and recent studies have shown that topotecan reduces HIF-1α expression, vessel density, and HIF-1 targets in U251-HRE xenografts (39).

Characterization of the U2OS-HRE-luc cells showed that HIF-1 activity was highly sensitive to reoxygenation. Although not surprising, this was in contrast to the observations obtained with a U251-HRE cell-based assay described previously, where a reoxygenation step was used before lysis (24). There is a possibility that the loss of PTEN in the glioma U251 cells (PTEN-null) may contribute to this effect. However, it is more likely that the difference between the U2OS-HRE-luc and U251-HRE systems is due to the different HRE-luciferase constructs used. Rapisarda et al. (24) used a construct where the SV40 promoter of pGL2 was replaced with a herpes simplex virus TK promoter fragment. Although this may have enabled the ability to assess HIF-1-mediated luciferase activity in the U251-HRE system even after significant reoxygenation of the cells, the resulting levels of HIF-1α protein were not assessed. It is most likely that HIF-1α protein expression would be significantly depleted by the reoxygenation of the U251-HRE cells. In contrast to our cell-based screen, the ability to assess and correlate the inhibition of HIF-1 activity with effects on HIF-1α protein expression would, therefore, not be possible within the screening parameters described by Rapisarda et al. (24).

Nevertheless, despite the differences in these independent screens, the observation that several identical hit compounds were identified suggests that the molecular targets involved in the induction of HIF-1 activity by the iron chelator DFX clearly overlap with those responsible for inducing HIF-1 activity in response to hypoxia. Indeed, it is clear that the prolyl hydroxylase enzymes, which target HIF-1α for hydroxylation and subsequent ubiquitination by VHL and degradation by the proteasome, require oxygen, ascorbate, 2-oxoglutarate, and, importantly, ferrous iron (40). The inactivation of the prolyl hydroxylase enzymes, which results in the stabilization of HIF-1α and subsequent activation of HIF-1, is therefore common to both hypoxia and DFX treatments.

We identified two novel compounds, NSC-134754 and NSC-643735, which were evaluated further. It was important to initially determine whether the compounds had any nonspecific activity and to establish whether they had an inhibitory effect on HIF-1 activity mediated by physiologic hypoxia, because our screen used DFX. This analysis was also carried out on NSC-607097, which was a hit identified in our screen and also by Rapisarda et al. (24). NSC-134754 and NSC-643735 did not significantly affect constitutive luciferase activity. Although NSC-607097 was found to have significant nonspecific activity, it was evaluated further because we concluded that it was a borderline compound in terms of its overall nonspecific activity.

We correlated the effects of the compounds on HIF-1 activity and HIF-1α expression in response to DFX, hypoxia, and IGF-1.

Figure 6. NSC-607097 inhibits HIF-1 activity but does not inhibit HIF-1α protein induction in response to DFX. U2OS-HRE-luc cells were treated with NSC-134754 (lane A), NSC-607097 (lane B), or NSC-643735 (lane C) over a range of concentrations or with vehicle control (DMSO) in the presence or absence of DFX (500 μmol/L) for 16 hours and then harvested. Samples were assayed for protein expression by Western blot analysis and luciferase activity. (A) Western blot analysis shows HIF-1α protein expression. Actin was used as a load control. (B) Graph shows luciferase reporter activity as a fold induction relative to the untreated vehicle control. Experiments were done in duplicate.
We found that NSC-607097 inhibited HIF-1 activity in DFX and hypoxia, and although NSC-607097 blocked the induction of HIF-1α in hypoxia, it did not significantly affect DFX-induced HIF-1α protein levels. Interestingly, while we found that NSC-643735 blocked DFX-induced HIF-1 activity and HIF-1α protein levels, NSC-643735 had no effect on HIF-1 activity or HIF-1α induction in response to hypoxic conditions, highlighting a mechanistic difference between the action of "chemical" hypoxia and physiologic hypoxia on the cells. Consistent with our observations, both NCS-134754 and NSC-607097 blocked the expression of Glut-1 protein in response to both DFX and hypoxia, whereas NCS-643735 had no effect on Glut-1 expression in hypoxia.

Importantly, all three compounds significantly inhibited IGF-1-induced HIF-1α expression. Taken together, our studies show that HIF-1α expression and HIF-1 activity can be differentially targeted and we have identified novel inhibitors with selective action for either blocking HIF-1α protein expression in response to hypoxia (NSC-134754) and/or IGF-1 (NSC-134754 and NSC-643735). The observation that NSC-134754 blocked both hypoxia and IGF-1-mediated HIF-1α induction, and also had limited nonspecific effects, suggests that this compound has particularly interesting properties that are HIF specific.

This study has shown that the U2OS-HRE-luc cell-based assay provides not only a robust method for identifying compounds

<table>
<thead>
<tr>
<th>Compound</th>
<th>DFX HIF-1α protein</th>
<th>DFX HIF-1 activity</th>
<th>Hypoxia HIF-1α protein</th>
<th>Hypoxia HIF-1 activity</th>
<th>IGF-1 HIF-1α protein</th>
<th>IGF-1 HIF-1 activity</th>
<th>IC50 growth inhibition (μmol/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>(A) NSC-134754</td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
<td>n.d.</td>
<td>0.5</td>
</tr>
<tr>
<td>(B) NSC-607097</td>
<td>+/-</td>
<td>+++</td>
<td>+</td>
<td>+++</td>
<td>+++</td>
<td>n.d.</td>
<td>0.1</td>
</tr>
<tr>
<td>(C) NSC-643735</td>
<td>++</td>
<td>++</td>
<td>--</td>
<td>--</td>
<td>+++</td>
<td>n.d.</td>
<td>100</td>
</tr>
</tbody>
</table>

NOTE: The effect of NSC-134754 (A), NSC-607097 (B), and NSC-643735 (C) at 10 μmol/L on HIF-1α and HIF-1 activity in response to DFX, hypoxia, and IGF-1 in U2OS-HRE-luc cells. ++, a compound that completely inhibits HIF-1α protein induction such that no protein is detected by Western analysis or inhibits HIF-1 activity by >80%. +++, a compound that completely inhibits HIF-1α protein induction or HIF-1 activity by >50%. +/-, a compound that inhibits HIF-1α induction or HIF-1 activity by <5%. --, a compound that has no significant inhibitory effect on HIF-1α induction or HIF-1 activity.

Abbreviation: n.d., not determined.
that inhibit DFX-induced HIF-1 activity, but also selects for compounds that are drug-like. The three hit compounds have molecular weights within the range of 334 to 418, Clog \( P \) values in the range 1.6 to 5.0, and have one to three hydrogen bond donors and four to seven hydrogen bond acceptors. These properties are all within the range considered to be preferable for drug development (41). Both NSC-134754 and NCS-643735 have interesting chemical properties; NSC-134754 is related to the benzoisoquinoline alkaloids known to be inhibitors of protein synthesis (30, 42), whereas NCS-643735 is a planar tetracyclic compound with potential for redox chemistry, originally prepared as a structural analogue of actinomycin D aglycone (31). Interestingly, NCS-607097, also known as DX-52-1 (32), has been investigated in a phase I clinical trial but the study was not continued due to toxicity (43). Because a cell-based assay approach was used to identify hits, the challenge is to establish how they inhibit HIF-1 activity. A thorough evaluation of the activity of the two novel compounds in a number of secondary assays is currently under way with the aim of defining their mechanism of action. Importantly, these studies will impact on their potential optimization for preclinical development and assessment in vivo. These studies may allow us to evaluate the contribution of converging signaling pathways on HIF and their respective relevance in HIF-dependent tumor progression and angiogenesis.

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Identification of Novel Small Molecule Inhibitors of Hypoxia-Inducible Factor-1 That Differentially Block Hypoxia-Inducible Factor-1 Activity and Hypoxia-Inducible Factor-1 α Induction in Response to Hypoxic Stress and Growth Factors


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