Noscapine inhibits hypoxia-mediated HIF-1α expression and angiogenesis *in vitro*: A novel function for an old drug

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Abstract. Overexpression of hypoxia-inducible factor-1 (HIF-1) is a common feature in solid malignancies related to oxygen deficiency. Since increased HIF-1 expression correlates with advanced disease stage, increased angiogenesis and poor prognosis, HIF-1 and its signaling pathway have become targets for cancer chemotherapy. In this study, we identified noscapine to be a novel small molecule inhibitor of the HIF-1 pathway based on its structure-function relationships with HIF-1 pathway inhibitors belonging to the benzylisoquinoline class of plant metabolites and/or to microtubule binding agents. We demonstrate that noscapine treatment of human glioma U87MG and T98G cell lines exposed to the hypoxic mimetic agent, $CoCl_2$, inhibits hypoxia-mediated HIF-1α expression and transcriptional activity as measured by decreased secretion of VEGF, a HIF-1 target gene. Inhibition of hypoxia-mediated HIF-1α expression was due, in part, to its ability to inhibit accumulation of HIF-1α in the nucleus and target it for degradation via the proteasome. One mechanism of action of microtubule binding agents is their antiangiogenic activity associated with disruption of endothelial tubule formation. We show that noscapine has similar properties *in vitro*. Thus, noscapine may possess novel antiangiogenic activity associated with two broad mechanisms of action: first, by decreasing HIF-1α expression in hypoxic tumor cells, upregulation of target genes, such as VEGF, would be decreased concomitant with its associated angiogenic activity; second, by inhibiting endothelial cells from forming blood vessels in response to VEGF stimulation, it may limit the process of neo-vascularization, correlating with antitumor activity *in vivo*. For more than 75 years, noscapine has traditionally been used as an oral cough suppressant with no known toxic side effects in man. Thus, the studies reported here have found a novel function for an old drug. Given its low toxicity profile, its demonstrated antitumor activity in several animal models of cancer and its potential to inhibit the HIF-1 pathway, noscapine should be considered as an antiangiogenic chemotherapy for glioma.

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Introduction

Overexpression of hypoxia-inducible factor-1 (HIF-1) is a common feature in solid malignancies and is related to oxygen deficiencies occurring via multiple mechanisms, including increased proliferation of tumor cells, lack of oxygen diffusion, decreased blood flow, and abnormal tumor vasculature. We and others have correlated increased HIF-1 expression with advanced disease stage, increased angiogenesis and poor prognosis (1-5). As a result, HIF-1 and its signaling pathway have become targets for cancer chemotherapy (6-12). A benefit of agents that affect HIF-1 activity or HIF-1α expression would be the prevention of activation of HIF-1 target genes, such as vascular endothelial growth factor (VEGF), which can contribute to tumor angiogenesis and promote tumor growth potential. Some clinical trials currently use anticancer compounds with pleiotropic effects, such as downregulation of HIF-1α expression, including the topoisomerase inhibitors, camptothecin and topotecan (13); the Hsp90 inhibitors, geldanamycin and 17-allyl-amino-geldanamycin (14-16); the redox protein inhibitor, thioredoxin inhibitor 1-methylpropyl-2-imidazolyl disulfide (17); the mammalian target of rapamycin (mTOR) kinase inhibitor, CCI-779 (18); the cyclin-dependent kinase inhibitor, flavopiridol (19); and the microtubule binding agent, 2-methoxyestradiol (2ME2) (20).

The discovery of novel small molecule inhibitors of HIF-1 has been the focus of much recent research using various high-throughput screening methods (13,21-24). These assays have identified several small molecules derived from natural products that inhibit the HIF-1 pathway at different points, including gene transcription, protein translation, post-translational modification and cellular localization (25). For example, chetomin, a fungal metabolite, binds to p300, thus disrupting its function and interaction with HIF-1α and preventing activation of the HIF-1 pathway (22). The compound, 103D5R, containing the 2,2-dimethylbenzopyran

structural motif found in many natural compounds, selected based on the fact that it ensures penetration into cells, decreased hypoxia-mediated HIF-1α protein expression and activation of target genes by reducing protein synthesis without affecting gene transcription (24). In another study, NSC-134754, a novel benzoisoquinoline alkaloid compound, was identified by its ability to inhibit hypoxia-mediated as well as growth factor-mediated induced HIF-1α expression (21). Most recently, two compounds derived from the aquatic plant *Saururus cernuus*, were found to be highly selective inhibitors of hypoxia-mediated HIF-1 activation by preventing nuclear accumulation of HIF-1α protein without affecting mRNA levels (23).

A major source of anticancer agents are natural products isolated from plants that have been used as medicinal plants in traditional folk medicine (26). Alkaloids, in particular the benzylisoquinoline class of plant metabolites, have been shown to have cytotoxic and antitumor properties that have potential to be developed as anticancer agents (27,28). In this study, we wished to test the potential of noscapine, a phthalide-isoquinoline non-narcotic alkaloid derived from opium (29,30), for its ability to inhibit hypoxia-mediated expression of HIF-1α in glioma cells. Since NSC-134754 and noscapine are related to each other by their common structure and the small molecule inhibitor, NSC-134754, was shown to inhibit HIF-1 activity and HIF-1α protein expression in response to hypoxia (21,28), we reasoned that noscapine might share a similar biological activity. An additional reason why noscapine might inhibit HIF-1 activity is based on a second known structure-function relationship. Noscapine's chemical structure is similar to the known microtubule binding agent, colchicine, and its binding activity to microtubules has been extensively characterized (29-31). Since the microtubule binding agents, 2ME2, paclitaxel and vincristine (all with different mechanisms of action on microtubule dynamics), have been shown to inhibit HIF-1α activity in response to hypoxia (20), we hypothesized that noscapine might also exhibit a similar activity.

In this study, we demonstrate that noscapine treatment of human glioma U87MG and T98G cell lines exposed to either hypoxia (1% O_2) or the hypoxic mimetic agent, $CoCl_2$, inhibits hypoxia-mediated HIF-1α expression and transcriptional activity as measured by decreased secretion of VEGF, a HIF-1 target gene. Inhibition of hypoxia-mediated HIF-1α expression was due, in part, to its ability to inhibit accumulation of HIF-1α in the nucleus and target it for degradation via the proteasome. One mechanism of action of microtubule binding agents, such as paclitaxel, is their antiangiogenic activity associated with disruption of endothelial tubule formation (32,33). In this report, we show that noscapine has similar properties *in vitro*. These results suggest that the antitumor activity demonstrated to date for the small molecule inhibitor, noscapine (29,30), may be associated with its dual properties to inhibit HIF-1 activity in response to hypoxic stress and to disrupt microtubule function.

Materials and methods

Cells and reagents. The human glioma cell lines, U87MG and T98G, were obtained from the American Type Culture Collection (ATTC; Manassas, VA, USA). Cells were cultured in a 5% CO_2 and 95% humidified air atmosphere at 37˚C in Dulbecco's modified Eagle's medium (DMEM) (Gibco BRL, Grand Island, NY, USA). For hypoxic exposure, cells were placed in a sealed modular incubator chamber (Billups-Rothenberg, Inc., Del Mar, CA) flushed with 1% O_2, 5% CO_2 and 94% N_2. Media were supplemented with 10% fetal bovine serum (FBS) (Atlanta Biologicals, Norcross, GA USA), 1% penicillin and streptomycin, and 2 mM glutamine (Gibco BRL, Grand Island, NY, USA). Cells were split every 3 days to ensure logarithmic growth. Noscapine hydrochloride (Nos), cobalt chloride ($CoCl_2$), cycloheximide (CHX), and myxothiazol were all purchased from Sigma (Sigma-Aldrich, Saint Louis, MO, USA). Noscapine was dissolved in dimethyl sulfoxide (DMSO) and stock solution (100 mM) was stored at -20˚C. $CoCl_2$ was prepared fresh in water for use at a final concentration of 125 μM. The 26S proteasome inhibitor, MG-262, purchased from Biomol (Plymouth Meeting, PA, USA), was dissolved in DMSO and stock solution (500 μM) was stored at -20˚C. The protein synthesis inhibitor, cycloheximide, was dissolved in DMSO and stock solution (100 μg/ml) was stored at 4˚C. Myxothiazol, an inhibitor of mitochondrial respiration, was dissolved in DMSO and stock solution (1 mM) was stored at -20˚C. Hydroethidine (HE), an oxidation-sensitive fluorescent probe, purchased from Molecular Probes (Eugene, OR, USA) was dissolved in DMSO and stock solution (300 μM) was stored at -20˚C.

Experimental culture conditions with $CoCl_2$ and proteasome inhibitor. Cells ($2x10^6$) were seeded in 10-cm dishes in 10 ml of complete growth medium for 24 h. To investigate HIF-1α expression, normoxic cells were cultured with 125 μM $CoCl_2$ for 5 h, in the absence or presence of noscapine (20-150 μM), or 500 nM MG-262. After incubation for 5 h, cells were harvested from each culture condition by washing three times with cold phosphate-buffered saline (PBS) and scraped into RIPA buffer (400 μl/plate). Lysates were incubated on ice for 1 h and clarified by centrifugation for 1 h at 4˚C and 16000 rpm and stored at -80˚C until quantitated for protein.

Western blot analysis. Cells were lysed in RIPA buffer (150 mM NaCl, 1% Nonidet P-40, 1% deoxycholate, 0.1% SDS, 10 mM Tris-HCl, pH 8.0, 1 mM EDTA, pH 8.0) supplemented with protease inhibitors (1 mM phenylmethylsulfonyl fluoride (PMSF), 0.2 mM sodium orthovanadate and 1 μg/ml aprotinin). Quantitation of protein was carried out using BCA reagent (Pierce, Rockford, IL, USA). Equal amounts of protein (30 μg) in the presence of 5% ß-mercaptoethanol (Sigma-Aldrich) were electrophoresed on 7.5% SDS-PAGE gels and subsequently transferred to Immobilon-P membranes (Millipore, Bedford, MA, USA) by electroblotting at 4˚C and 33 V overnight. Western blot analysis was performed as described (19) with the following antibodies: mouse anti-HIF-1α monoclonal antibody used at 1:1000 (clone 54, BD Transduction Laboratories, San Jose, CA, USA), mouse anti-actin monoclonal antibody used at 1:50,000 (clone C4, Chemicon International, Inc., Temecula, CA, USA). Sheep anti-mouse IgG (Amersham Life Pharmacia Biotech, Piscataway, NJ, USA) horseradish peroxidase-conjugated secondary antibodies were used at 1:2000. Immuno-

detection was carried out using either the Supersignal West Femto (HIF-1α) or Pico (actin) Maximum Sensitivity Substrate ECL detection system (Pierce Biotechnology Inc., Rockford, IL, USA). Visualization and quantitation of protein bands was performed using Fuji medical X-ray film (Super Rx, Fuji Photo Film, Co., Tokyo) with Quantity One (Version 4.2.1) or NIH image software (Version 1.62).

Immunofluorescent microscopy. Cells ($3x10^4$) were seeded onto poly-D-lysine coated glass coverslips and incubated overnight. Cells were untreated or exposed to 125 μM $CoCl_2$ for 24 h, in the absence or presence of 50 μM noscapine and processed for immunofluorescence. For HIF-1α immunofluorescence, cells were fixed in 4% paraformaldehyde in PBS for 15 min, then washed 5 min in PBS, permeabilized for 10 min in 0.5% Igepal (Sigma-Aldrich) followed by a final wash of PBS for 5 min; all steps were performed at room temperature. Non-specific binding was blocked by incubation in blocking buffer containing 2% bovine serum albumin (BSA) in PBS for 30 min at room temperature. Cells were incubated overnight at 4˚C with primary mouse monoclonal anti-HIF-1α antibody (BD Transduction Labs. #610959) diluted 1:200 in blocking buffer. Cells were washed in blocking buffer three times for 5 min each before incubation with secondary donkey anti-mouse FITC-conjugated antibody (Jackson Immunoresearch, West Grove, PA, USA) diluted 1:300 in blocking buffer for 2 h at room temperature in the dark. For tubulin immunofluorescence, cells were fixed in 100% methanol for 10 min at -20˚C. Cells were incubated in blocking buffer for 30 min at 37˚C followed by incubation for 2 h at 37˚C with FITC-conjugated monoclonal anti-α-tubulin antibody (clone DM 1A, Sigma-Aldrich) diluted 1:500 in blocking buffer in the dark. After three washes in PBS, cells were counterstained with propidium iodide (PI, 20 μg/ml) or DAPI (100 ng/ml) for 20 min at room temperature. After washing three times in PBS, coverslips were mounted onto Fisher ColorFrost glass slides using the ProLong antifade kit (Molecular Probes, Eugene, OR, USA). Images were captured using a Nikon Fluorescent microscope and adjusted using Adobe Photoshop 7.0 software.

Isolation and analysis of RNA. Total RNA was isolated from monolayers of U87MG cells using the RNeasy kit (Qiagen Inc, Valencia, CA, USA) according to the manufacturer's directions. RNA was quantitated by absorbance at 260 nm. For reverse transcription, 2 μg of total RNA was reverse-transcribed using SuperScript II RNase H reverse transcriptase (Invitrogen, Carlsbad, CA, USA) and random hexamer primers (Invitrogen) at 25˚C for 10 min and 42˚C for 1 h for cDNA synthesis. Two μl of the reverse transcription product was used as a template for PCR amplification. PCR was performed under standard conditions in a 50 μl reaction mix containing 1X PCR buffer, 1 unit of Platinum Taq polymerase (Invitrogen), 200 μM dNTP mix, 1.5 mM $MgCl_2$, 100 nM of HIF-1α primers (5'-GTCGGACAGCCTCACCA AACAGAGC-3'-sense, 5'-GTTAACTTGATCCAAAGCTC TGAG-3'-antisense) or 25 nM of ß-actin primers (5'-GTA CCACTGGCATCGTGATGGACT-3'-sense, 5'-ATCCACA CGGAGTACTTGCGCTCA-3'-antisense). The PCR conditions consisted of a 3-min initial denaturation step (95˚C) followed by 25 cycles of denaturation (95˚C, 30 sec), annealing (55˚C, 30 sec), and extension (72˚C, 50 sec), followed by a final elongation step of 7 min at 72˚C. Twenty μl of PCR product was analyzed on 3% agarose gels stained with ethidium bromide. Quantitation of bands was performed using Bio-Rad Fluor-S apparatus (BioRad, Hercules, CA) with Quantity One (Version 4.2.1) software.

Determination of the half-life of HIF-1α. To determine the half-life of HIF-1α protein, cells were treated with cycloheximide (CHX) to block protein synthesis. Cells were grown under normoxia alone or with 125 μM $CoCl_2$ for 3 h to induce HIF-1α protein expression. Then, cultures were washed twice with fresh media to remove $CoCl_2$ and incubated with cycloheximide (100 μg/ml) in the absence or presence of noscapine (150 μM). Cultures were harvested at 30 and 60 min and lysates were collected for Western blot analysis of HIF-1α protein expression.

VEGF quantification. VEGF protein released into the conditioned medium was measured using a commercial ELISA kit (R&D System, Minneapolis, MN) according to the manufacturer's instructions. Cells ($5x10^5$) were seeded in 6-well plates in 2 ml complete growth medium. After 24 h, the cells were washed twice with PBS and pre-conditioned for 1 h at 37˚C in 1 ml DMEM containing 2% FBS. The pre-conditioned medium was replaced with 1 ml DMEM containing 2% FBS alone or with 125 μM $CoCl_2$ in the absence or presence of 150 μM noscapine. After 24 h incubation to allow VEGF protein secretion under the various culture conditions, media were collected and 1 mM PMSF was added. The supernatant was clarified by centrifugation for 5 min at 16000 rpm, aliquoted and stored at -80˚C until quantification for VEGF. The assay was run in triplicate and repeated twice with similar results. Data from two independent experiments were pooled for statistical analysis.

Determination of ROS. The generation of reactive oxygen species (ROS) was determined fluorometrically using hydroethidine (HE; Molecular Probes, Eugene, OR, USA). This oxidation-sensitive fluorescent probe reacts predominantly with O_2. At each time point studied, cells (10^6/ml) were incubated in Hepes-buffered RPMI-1640 medium with 3 μM HE for 15 min and samples were analyzed on a Becton Dickinson FACScan (San Jose, CA, USA) or by fluorescent microscopy. Excitation and emission wavelengths were 488 and 610 nm, respectively. ROS levels, as determined by fluorescence of control cells, served as a baseline for determination of increased ROS levels in response to exposure to noscapine (100, 150 μM). Myxothiazol (1 μM), an inhibitor of mitochondrial respiration, was used as a positive control for generating ROS in U87MG cells. For microscopy detection of ROS levels, an aliquot of cells ($5x10^4$) was attached to poly-D-lysine coated glass slides using a cytospin (400 rpm, 10 min) and images were captured using a Nikon Fluorescent microscope and adjusted using Adobe Photoshop 7.0 software.

In vitro tubule formation assay. Human umbilical vein endothelial cells (HUVECs) were used to assess the antiangiogenic potential of the drug, noscapine. HUVECs (Cambrex Bio

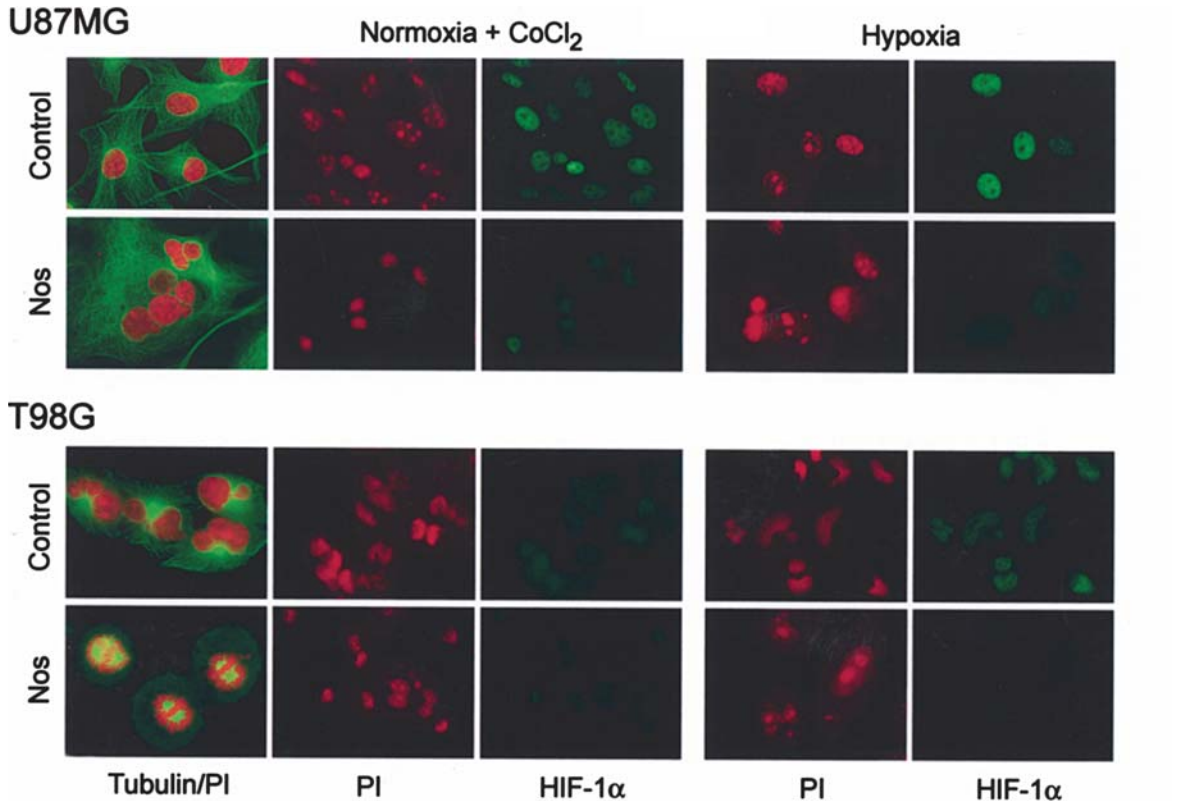

Figure 1. Effect of noscapine on hypoxia-mediated accumulation of HIF-1α in the nucleus. U87MG or T98G cells were seeded onto poly-D-lysine coated glass coverslips and incubated overnight. Cells were untreated or exposed to 1% O_2 or 125 μM $CoCl_2$ for 24 h, in the absence or presence of 50 μM noscapine and processed for immunofluorescence. Cells were immunostained with an antiserum specific for HIF-1α (green fluorescence) or tubulin (green fluorescence) and counterstained with propidium iodide (PI, red fluorescence) to visualize the nuclei. Images were captured using Nikon Fluorescent microscope and adjusted using Adobe Photoshop 7.0 software (x40).

Science, Baltimore, MD, USA) were seeded ($2x10^5$/well) on Matrigel-coated wells (24-well plate) in the absence or presence of different doses of noscapine. The extent of tubule formation was quantified. A magnification x10 was used to count the number of branched tubules within 4 viewing fields per insert. Cultures were photographed at 16 h using a digital camera attached to an inverted phase-contrast microscope. The assay was run in duplicate and repeated two times with similar results. Data from two independent experiments were pooled for statistical analysis. Data are expressed as a percentage of the control (number of branched tubules in treated cultures/number of branched tubules in untreated cultures) x 100 (mean ± SE).

Statistical analysis. Experiments were performed at least two or three times and determinations were performed in replicates. Results are expressed as mean ± SE. All analyses for the conditions being compared were performed using a two-sided Student's t-test for significance ($P<0.05$). All analyses used StatView software (SAS Inst, Cary, NC).

Results

Effect of noscapine on hypoxia-mediated accumulation of HIF-1α in the nucleus. First, we tested the ability of noscapine to inhibit HIF-1 activity by evaluating levels of HIF-1α protein expressed in the nuclei of glioma cells exposed to different types of hypoxic stress. We treated cells with hypoxia (1%) or the hypoxic mimetic agent, $CoCl_2$, as described previously (14,19). U87MG and T98G cells were seeded onto glass coverslips. On the following day, the cells were untreated or exposed to 1% oxygen or $CoCl_2$ for 24 h, in the absence or presence of 50 μM noscapine and processed for immunofluorescent microscopy (Fig. 1). As described previously, noscapine induces the polymerization and stabilization of microtubules resulting in mitotic arrest and multinucleation (29,30). Immunostaining with anti-α-tubulin antibody (green) showed normal microtubule arrays in both of the untreated glioma cells. However, noscapine treatment of U87MG cells (upper panel) induced giant multinucleated cells compared with T98G cells (lower panel) that did not show multinucleation but did show abnormal multipolar spindles, reported previously for noscapine-treated HeLa cells (31).

Treatment of U87MG or T98G cells with 1% oxygen or $CoCl_2$ for 24 h induced accumulation of HIF-1α protein within the nucleus (green signal). Noscapine treatment induced a decrease in the nuclear accumulation of HIF-1α protein under both conditions of hypoxic stress. PI staining was used to reveal the DNA within the nuclei (red signal). For all subsequent experiments performed in this report, $CoCl_2$ treatment was used to induce hypoxia-mediated HIF-1α protein expression in glioma cells grown under normoxic conditions.

Effect of noscapine on the proteasomal degradation pathway. To test whether inhibition of HIF-1α protein expression

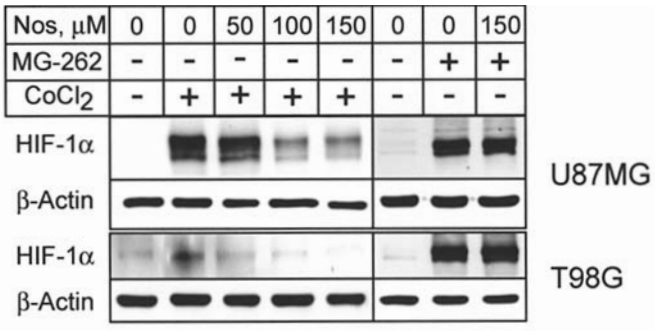

Figure 2. Effect of noscapine on the proteasomal degradation pathway. U87MG or T98G cells were plated in 10-cm dishes. After 24 h, cells were untreated or exposed to 125 μM $CoCl_2$ or 500 nM MG-262 for 5 h in the absence or presence of different concentrations of noscapine (50-150 μM). Cells were harvested after 5 h and lysates were immunoblotted to detect levels of HIF-1α expression. ß-actin was used as the loading control. Representative Western blot results are shown from one of two independent experiments. Protein bands were quantitated by densitometry.

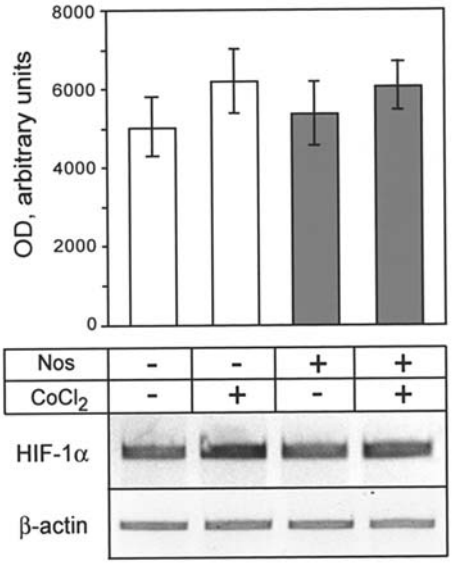

Figure 3. Effect of noscapine on hypoxia-mediated HIF-1α mRNA expression. U87MG cells were plated in 10-cm dishes. After 24 h, cells were untreated or exposed to 125 μM $CoCl_2$ for 5 h in the absence or presence of 150 μM noscapine. Cells were harvested after 5 h and total RNA was extracted for gene expression studies. Levels of HIF-1α and ß-actin gene expression were measured using a semi-quantitative RT-PCR assay. Samples in each assay were run in duplicate. PCR products were analyzed on 3% agarose gels stained with ethidium bromide. DNA bands were quantitated by densitometry. Results of one representative PCR assay are shown from four independent experiments. Bar graphs represent the pooled data from the experiments comparing relative levels of HIF-1α expression in the different treatment groups. Error bars indicate the range of the determinations (mean ± SE).

induced by noscapine was associated with degradation of HIF-1α via the proteasome pathway, we assessed the effect of the 26S proteasome inhibitor, MG-262. In the absence of $CoCl_2$ treatment, the cell lines showed baseline levels of HIF-1α expression. Treatment with $CoCl_2$ upregulated HIF-1α expression approximately 30-fold in U87MG cells and 2-fold in T98G cells compared with the respective untreated control culture (Fig. 2, lane 2 vs 1). Simultaneous addition of noscapine with $CoCl_2$ to the cultures inhibited HIF-1α expression in a dose-dependent fashion. Treatment with 150 μM noscapine reduced HIF-1α expression by approximately 60% in U87MG cells and by 90% in T98G cells (Fig. 2, lane 2 vs 5).

Since HIF-1α expression is mediated, in part, by 26S proteasomal degradation, inhibition of proteasome function results in the accumulation of HIF-1α protein in cells grown under normoxic conditions (34). As expected, the glioma cell lines exposed to the proteasome inhibitor, MG-262, showed increased levels of expression of HIF-1α by approximately 10-fold in U87MG cells and by 20-fold in T98G cells (Fig. 2, lane 6 vs 7). The reduction of HIF-1α expression by noscapine treatment was blocked when the function of the 26S proteasome was inhibited (Fig. 2, lane 7 vs 8), suggesting that noscapine promoted degradation of HIF-1α, in part, through the proteasome degradation pathway.

Effect of noscapine on hypoxia-mediated HIF-1α mRNA expression. Since regulation of the HIF-1 pathway can be controlled at both the transcriptional and translational levels, we next investigated whether noscapine treatment affected HIF-1α gene transcription. U87MG cells were grown under normoxic conditions for 5 h in the absence (Fig. 3, lanes 1 and 3) or presence of $CoCl_2$ (Fig. 3, lanes 2 and 4) and in the absence (Fig. 3, lanes 1 and 2) or presence of 150 μM noscapine (Fig. 3, lanes 3 and 4). Cells were harvested after 5 h and total RNA was extracted for gene expression studies. Representative results of one of three independent PCR assays together with the relative expression levels of HIF-1α in each treatment group are shown (Fig. 3). Treatment with $CoCl_2$ increased HIF-1α mRNA relative to the untreated culture (Fig. 3, lanes 1 vs 2) as we have described previously (19). However, noscapine treatment had no significant effect on HIF-1α mRNA levels regardless of whether $CoCl_2$ was absent or present (Fig. 3, lanes 3 vs 4).

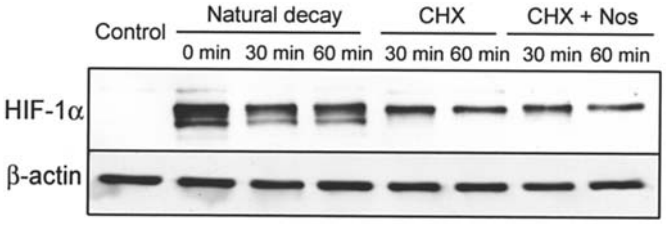

Figure 4. Effect of noscapine on the half-life of HIF-1α protein. U87MG cells were plated in 10-cm dishes. After 24 h, cells were untreated or exposed to 125 μM $CoCl_2$ for 3 h to induce HIF-1α protein expression. Cells were washed to remove $CoCl_2$ and cycloheximide (CHX, 100 μg/ml) was added in the absence or presence of 150 μM noscapine. Cells were harvested after 30 and 60 min and lysates were immunoblotted to detect levels of HIF-1α protein. ß-actin was used as the loading control. Results of one representative Western blot are shown from one of two independent experiments. Protein bands were quantitated by densitometry.

Effect of noscapine on the half-life of HIF-1α protein. We next tested the effect of noscapine on the stability of HIF-1α protein in U87MG cells. To assess changes in HIF-1α stability, general protein synthesis was blocked by cycloheximide treatment. Representative results of one of two independent

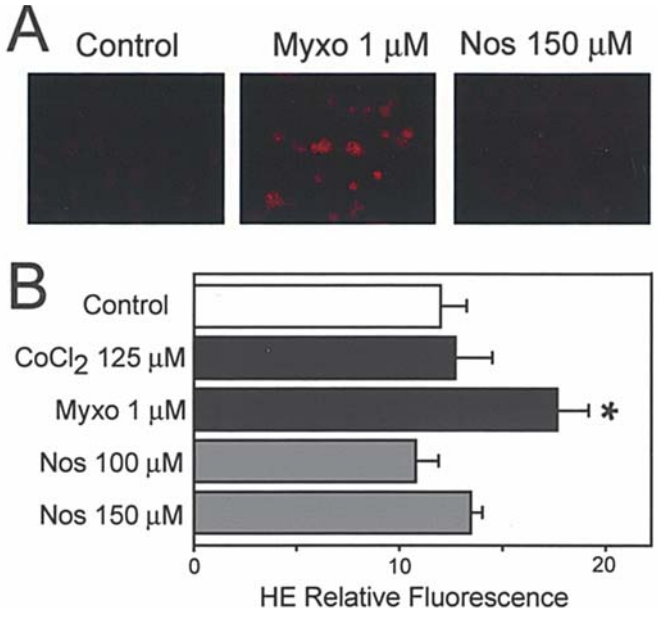

Figure 5. Effect of noscapine on generation of ROS. U87MG cells were plated in 60-mm dishes. After 24 h, cells were untreated or exposed to $CoCl_2$, noscapine (Nos, 100 or 150 μM) or 1 μM myxothiazol (Myxo) for 5 h and samples were collected by trypsinization and incubated with 3 μM HE at 37˚C for 15 min. (A) An aliquot of cells was cytospun onto poly-D-lysine coated slides and images were captured using a Nikon Fluorescent microscope and adjusted using Adobe Photoshop 7.0 software (x40). (B) The remaining cells were analyzed for mean HE fluorescence by flow cytometry. Bar graphs represent the pooled data from three independent experiments comparing relative fluorescence levels of ROS expression in the different treatment groups. *A statistically significant difference between the control and myxothiazol-treated groups.

experiments are shown (Fig. 4). U87MG cells were first stimulated with $CoCl_2$ for 3 h to induce HIF-1α expression. HIF-1α expression was upregulated approximately 10-fold compared with the untreated control culture (Fig. 4, lane 2 vs 1). Cultures were washed to remove $CoCl_2$ and then incubated with cycloheximide (CHX) in the absence or presence of noscapine for up to 1 h. The natural decay of HIF-1α protein expression decreased by 55% over the 60-min interval. As expected, inhibition of protein synthesis by cycloheximide treatment greatly decreased HIF-1α expression compared with natural decay. However, the half-life of HIF-1α protein did not change in the absence or presence of noscapine treatment (Fig. 4, lanes 5 and 6 vs 7 and 8) as the relative ratio of of HIF-1α:ß-actin remained unchanged.

Effect of noscapine on generation of ROS. Although we have explored several mechanisms by which noscapine might inhibit HIF-1α expression, including whether its effect was through increased degradation of HIF-1α, decreased gene transcription or decreased protein synthesis, we wanted to exclude the possibility that noscapine induced reactive oxygen species (ROS). When the electron transport of mitochondria is blocked, ROS is produced, which can lead to the rapid degradation of HIF-1α under hypoxic conditions (35). This is due to increased levels of intracellular O_2 and reactivation of prolyl hydroxylase activity resulting in the ubiquitination and subsequent degradation of HIF-1α. In view of the fact that the microtubule binding agent, 2ME2, produced ROS that was associated with HIF-1α degradation (36), we next tested whether treatment of U87MG cells with noscapine could induce ROS. U87MG cells were untreated or treated with $CoCl_2$, noscapine or myxothiazol for 5 h. Cells were collected and incubated with hydroethidine and examined both by FACS analysis and fluorescent microscopy. Representative results of hydroethidine fluorescence (red) (Fig. 5A) are shown together with quantitative FACS data pooled from three independent experiments (Fig. 5B). Treatment with $CoCl_2$ or high doses of noscapine alone had little effect on hydroethidine fluorescence. However, treatment with myxothiazol, an inhibitor of mitochondrial respiration, resulted in an increase in relative fluorescence detected by fluorescent microscopy compared with untreated control cells or cells treated with 150 μM noscapine (Fig. 5A). Relative fluorescence units measured by FACS analysis showed a significant increase with myxothiazol treatment ($P<0.02$) compared with the control cells (Fig. 5B). Thus, the microtubule binding agent noscapine does not appear to produce ROS as reported for the microtubule binding agent, 2ME2 (36).

Effect of noscapine on VEGF production. Since increased expression of HIF-1α protein in the nucleus promotes transcriptional activation of many target genes, one of which is vascular endothelial growth factor (VEGF), we next determined whether noscapine treatment reduced VEGF secretion concomitant with reduced HIF-1α expression. VEGF protein secreted into the conditioned medium by U87MG and T98G cells was measured by ELISA as we have described (19). Results pooled from two independent experiments are shown (Fig. 6). $CoCl_2$ treatment induced a 2-fold increase in VEGF compared with untreated cells that showed a low basal level of VEGF secretion under normoxic conditions. Simultaneous treatment with $CoCl_2$ and 150 μM noscapine significantly reduced VEGF secretion in U87MG ($P<0.005$) and T98G cells ($P<0.01$).

Effect of noscapine on endothelial cell tubule formation in vitro. Tumor vasculature is an important target for anticancer drugs as it is intimately associated with providing tumor growth promoting potential. Recent attention has focused on the role of microtubule binding agents, such as paclitaxel (Taxol) and docetaxel (Taxotere), in affecting endothelial cell function (32,33). The tube formation assay measures the ability of HUVECs to spontaneously form capillary-like structures on Matrigel. In the absence of drug treatment, HUVECs undergo alignment and formation into cords, which establishes the pattern for tubule formation. HUVECs were seeded on Matrigel-coated wells in the absence or presence of different doses of noscapine. The results of one representative tubule formation assay (Fig. 7A), together with quantitative data pooled from two independent experiments are shown (Fig. 7B). After incubation for 16 h, the cords fuse into continuous tubules with a complete lumen to form capillary-like structures. In the presence of noscapine, tubule formation was inhibited in a dose-dependent manner, with a 30% reduction in tubule formation observed at 50 μM and a 40% decrease at both of the higher drug concentrations. Noscapine treatment of HUVECs significantly reduced the number of branched tubes compared with untreated cultures ($P<0.005$).

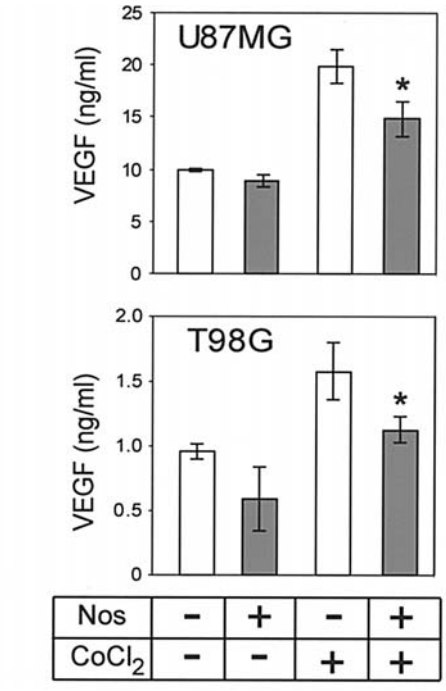

Figure 6. Effect of noscapine on VEGF production. VEGF protein released into the conditioned medium was measured using a commercial ELISA kit. U87MG or T98G cells were seeded in 6-well plates in 2 ml of growth medium. The assay was run in triplicate and repeated twice. Bar graphs represent data pooled from the experiments for statistical analysis comparing VEGF secretion in cultures with and without $CoCl_2$ and noscapine treatment. Error bars indicate the range of the determinations (mean ± SE). *A statistically significant difference between the two $CoCl_2$-treated groups.

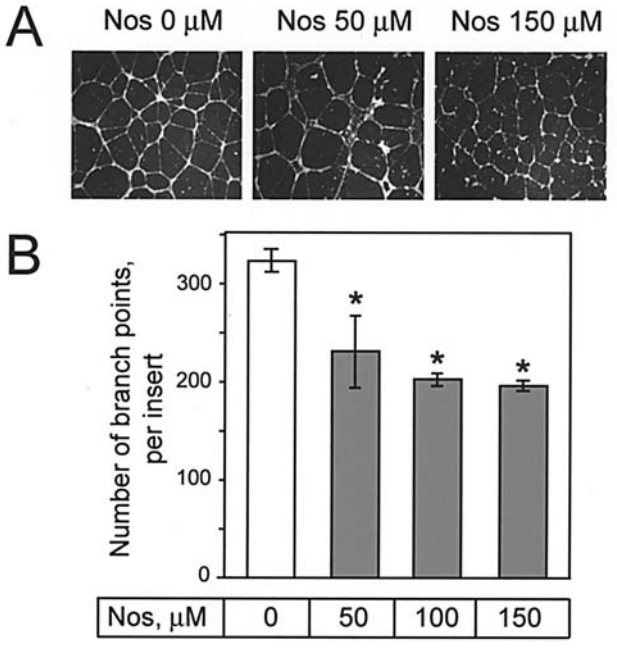

Figure 7. Effect of noscapine on endothelial cell tubule formation *in vitro*. HUVECs were seeded on Matrigel-coated wells in the absence or presence of different doses of noscapine. The assay was run in duplicate and repeated twice. (A) Representative images of tubule formation at 16 h were captured using a Nikon Fluorescent microscope and adjusted using Adobe Photoshop 7.0 software (x40). (B) Bar graphs represent pooled data from the experiments for statistical analysis comparing number of branched tubes in cultures with and without noscapine treatment. *A statistically significant difference between the control and noscapine-treated groups.

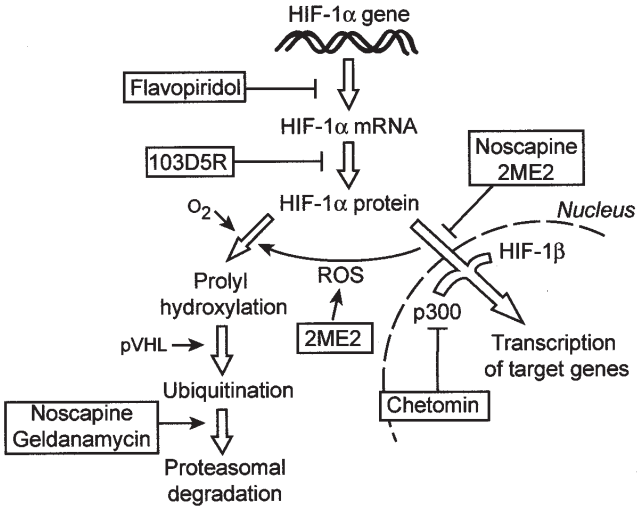

Figure 8. Inhibition of the HIF-1 pathway by anticancer drugs. HIF-1 is a heterodimeric transcription factor consisting of the HIF-1α and HIF-1ß subunits. The availability of oxygen (O_2) regulates HIF-1 expression and transcription of hypoxia-regulated genes involved in the adaptation of cells to a hypoxic microenvironment. Several anticancer compounds have been shown to target HIF-1 activity or HIF-1α expression by different mechanisms acting at different points in the HIF-1 pathway, including gene transcription, protein translation, posttranslational modification and cellular localization, as shown in the schema. The cyclin-dependent kinase inhibitor, flavopiridol, inhibits HIF-1α gene transcription (19). Compound 103D5R reduces HIF-1α protein synthesis inhibiting HIF-1α protein expression and subsequent activation of hypoxia-regulated target genes (24). Formation of reactive oxygen species (ROS) by the anticancer agent, 2ME2, can provide a source of intracellular O_2 within hypoxic cells that can reactivate prolyl hydroxylases resulting in ubiquitination of HIF-1α and its degradation via the 26S proteasome (36). Both of the microtubule binding agents, noscapine and 2ME2, block the accumulation of HIF-1α protein in the nucleus (20). Chetomin inhibits binding of the co-activator p300 to HIF-1 decreasing HIF-1α/p300 complex formation, preventing activation of the HIF-1 pathway (22). The Hsp90 inhibitor, geldanamycin, and the microtubule binding agent, noscapine, both promote degradation of hydroxylated and ubiquitinated HIF-1α protein by the 26S proteasome (14-16). Thus, there are multiple ways in which anticancer agents can affect HIF-1 activity to limit hypoxia-mediated tumor growth promoting potential.

Discussion

In this study, we identified noscapine to be a novel small molecule inhibitor of the HIF-1 pathway based on its structure and/or functional relationships with two other recently identified HIF-1 pathway inhibitors (20,21). The first compound, NSC-134754, belongs to the benzylisoquinoline class of alkaloid plant metabolites that have shown promising anticancer properties (21,27). It was discovered in a high-throughput screening assay designed to identify compounds that inhibited HIF-1 activity with a corresponding reduction in HIF-1α protein expression induced by deferoxamine mesylate (DFX), a hypoxia mimetic. However, NSC-134754 also inhibited HIF-1 activity induced by the relevant physiological stimulus of low oxygen (1% O_2), compared with another novel compound, NSC-643735, identified in the same screen that could not, by measuring expression levels for the hypoxia-inducible HIF-1 target gene, Glut-1 (21). The differential regulation of HIF-1 activity by these two small molecule inhibitory compounds to hypoxic stress underscores the fact that there may be important

differences between signaling pathways induced in cells by true hypoxia (1% O_2) versus the use of hypoxia mimetics, such as the DFX or cobalt chloride ($CoCl_2$) used in this study.

Our results show that the inhibitory action of noscapine on HIF-1α protein expression in glioma cells exposed to hypoxic stress induced either by hypoxia (1% O_2) or by the hypoxic mimetic, $CoCl_2$, was identical to that reported for NSC-134754. For the noscapine studies, we used decreased expression of the HIF-1 target gene product VEGF as our readout to monitor the inhibition of HIF-1 transcriptional activity. In summary, two different novel small molecule inhibitors of HIF-1, NSC-134754 and noscapine, members of the benzylisoquinoline class of compounds, have shown similar inhibitory functions on cells responding to hypoxic stress factors. This capacity to disrupt the HIF-1 pathway makes them potential candidates for preclinical testing in animal models, such as we have described for the pleiotropic cyclin-dependent kinase inhibitor, flavopiridol, which inhibits hypoxia-mediated HIF-1α expression in glioma cells *in vitro* and the angiogenic activity of glioma tumors *in vivo* (19,37).

A second compound, 2-methoxyestradiol (2ME2), was discovered to inhibit HIF-1 activity by disrupting the function of microtubules in the cytoskeleton, preventing accumulation of HIF-1α protein in the nucleus and transcriptional activation of the VEGF gene (20). As mentioned previously, noscapine was identified as a potential microtubule binding agent by comparing its chemical structure with the chemical structures of known microtubule binding compounds derived from natural products, such as colchicine and podophyllotoxin (30). Given the fact that vincristine and paclitaxel, two other microtubule binding drugs that either destabilize or stabilize microtubules respectively, also inhibited HIF-1α protein from translocating to the nucleus as 2ME2, we asked whether this might be a common mechanism of action for this class of drugs. Therefore, we tested noscapine under both conditions of hypoxic stress and found that noscapine treatment of glioma cells, which stabilizes microtubules similar to paclitaxel, was also able to inhibit accumulation of HIF-1α in the nucleus. This inhibition was associated with decreased production of VEGF, our readout gene product for measuring HIF-1 transcriptional activity.

Since the HIF-1 pathway can be inhibited at several different points (Fig. 8), including posttranslational modification, gene transcription, or protein translation, we investigated whether noscapine had any effect on proteasomal degradation of HIF-1α protein, or synthesis of its mRNA or protein. We found that noscapine promoted the degradation of HIF-1α protein via the proteasome. This conclusion was supported by the finding that the proteasome inhibitor, MG-262, not only induced the accumulation of HIF-1α protein in cells grown under normoxic conditions, as would be expected, but also prevented noscapine-induced decrease in HIF-1α protein expression. Noscapine treatment also had no effect on gene transcription or protein synthesis. Because certain known HIF-1 inhibitory drugs, such as 2ME2, have been shown to block mitochondrial electron transport that produces reactive oxygen species (ROS) inside the cell that can serve to rapidly degrade HIF-1α under hypoxic conditions, we asked whether noscapine also induced ROS (36). Our results showed that noscapine treatment, even at the highest dose of 150 μM, did not induce ROS. In summary, inhibition of HIF-1 and the induction of HIF-1α protein by noscapine can be attributed to a posttranslational mechanism involving degradation by the proteasome pathway similar to that described for the Hsp90 inhibitor, geldanamycin (16). These results contrast with our findings for the cyclin-dependent kinase inhibitor, flavopiridol, which inhibited HIF-1α by a proteasome-independent pathway, mainly at the level of gene transcription (19). As novel small molecule inhibitors of HIF-1, such as noscapine, continue to be discovered and their mechanisms of action understood, they will be added to a growing list of compounds reported to inhibit HIF-1α protein, recently summarized in a review by Powis and Kirkpatrick (9).

For microtubule binding agents, the mechanisms of action also are becoming better understood (38,39). Taxanes, such as paclitaxel, show antiangiogenic properties due to their ability to target endothelial cell proliferation, migration and differentiation into capillary-like tubes, processes required for new blood vessel formation to supply a growing tumor (32,33). In view of the fact that noscapine treatment of glioma tumor cells inhibited their secretion of the potent angiogenic factor, VEGF, associated with HIF-1 transcriptional activity, we also wanted to determine whether noscapine, similar to other microtubule binding agents such as taxanes, would block the ability of HUVECs to spontaneously form capillary-like structures on Matrigel. Our results showed that noscapine was a potent inhibitor of tubule formation. Although the antiangiogenic potential of noscapine has not been directly evaluated, it has demonstrated antitumor activity in several animal models (29,30,40,41). Most recently, noscapine was shown to cross the blood-brain barrier and inhibit the intracerebral growth of rodent brain tumors (42). Thus, noscapine may possess novel antiangiogenic activity associated with two broad mechanisms of action: first, by decreasing HIF-1α expression in tumor cells within a hypoxic tumor microenvironment, upregulation of target genes, such as VEGF, would be decreased concomitant with its associated angiogenic activity; second, by inhibiting endothelial cells from forming blood vessels in response to VEGF stimulation, it may limit the process of neo-vascularization, correlating with antitumor activity *in vivo*.

We have shown that noscapine, a member of the benzylisoquinoline class of alkaloids and a tubulin binding agent, inhibits hypoxia-mediated HIF-1α expression and angiogenesis *in vitro*. For more than 75 years, noscapine has traditionally been used as an oral cough suppressant with no known toxic side effects in man (29,30). More recently, noscapine has shown efficacy in the treatment of stroke patients (43). Thus, the studies reported here have found a novel function for an old drug. Given its low toxicity profile, its demonstrated antitumor activity in several animal models of cancer, including an animal model of glioma and its potential to inhibit the HIF-1 pathway, noscapine should be considered as an antiangiogenic chemotherapy for glioma, one of the most angiogenic tumors known.

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