Identification of a Novel Small-Molecule Inhibitor of the Hypoxia-Inducible Factor 1 Pathway

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Abstract

Hypoxia-inducible factor 1 (HIF-1) is the central mediator of cellular responses to low oxygen and has recently become an important therapeutic target for solid tumor therapy. Inhibition of HIF-1 is expected to result in the attenuation of hypoxia-inducible genes, which are vital to many aspects of tumor biology, including adaptative responses for survival under anaerobic conditions. To identify small molecules inhibiting the HIF-1 pathway, we did a biological screen on a 10,000-membered natural product-like combinatorial library. The compounds of the library, which share a 2,2-dimethylbenzopyran structural motif, were tested for their ability to inhibit the hypoxic activation of an alkaline phosphatase reporter gene under the control of hypoxia-responsive elements in human glioma cells. This effort led to the discovery of 103D5R, a novel small-molecule inhibitor of HIF-1α. 103D5R markedly decreased HIF-1α protein levels induced by hypoxia or cobaltous ions in a dose- and time-dependent manner, whereas minimally affecting global cellular protein expression levels, including that of control proteins such as HIF-1β, IκBα, and β-actin. The inhibitory activity of 103D5R against HIF-1α was clearly shown under normoxia and hypoxia in cells derived from different cancer types, including glioma, prostate, and breast cancers. This inhibition prevented the activation of HIF-1 target genes under hypoxia such as vascular endothelial growth factor (*VEGF*) and glucose transporter-1 (*Glut-1*). Investigations into the molecular mechanism showed that 103D5R strongly reduced HIF-1α protein synthesis, whereas HIF-1α mRNA levels and HIF-1α degradation were not affected. 103D5R inhibited the phosphorylation of Akt, Erk1/2, and stress-activated protein kinase/c-jun-NH_2-kinase, without changing the total levels of these proteins. Further studies on the mechanism of action of 103D5R will likely provide new insights into its validity/applicability for the pharmacologic targeting of HIF-1α for therapeutic purposes. (Cancer Res 2005; 65(2): 605-12)

Introduction

The development of intratumoral hypoxia and hypoglycemia is a hallmark of rapidly growing solid tumors. Increased tumor energy demands and oxygen consumption are not met by a poorly functional tumor vasculature that renders insufficient blood supply due to vascular occlusion, regression, thrombosis, arteriovenous shunts, and leakage. The partial oxygen tension in normal tissues ranges from 30 to 60 mm Hg, whereas in human tumors it is mostly below 10 mm Hg or even <5 mm Hg (equivalent to 0.7%; ref. 1). The presence of hypoxia in tumors plays a negative role in patient prognosis. Hypoxic tumor cells are resistant to conventional chemotherapy and radiotherapy and hypoxia promotes a more malignant phenotype (1). It is therefore rational to target the hypoxic regions of tumors or disrupt events initiated by hypoxia.

A key mediator expressed in many cell types in response to oxygen deprivation is hypoxia-inducible factor 1 (HIF-1), a heterodimeric transcription factor that consists of an oxygen-regulated HIF-1α subunit and a constitutively expressed aryl hydrocarbon receptor nuclear translocator (also called HIF-1β; ref. 2). The binding of HIF-1 along with coactivators, including p300/CBP, SRC-1, and TIF2 to hypoxia-responsive elements (HRE) leads to transcriptional activation of a variety of genes involved in angiogenesis, oxygen transport, glycolysis, glucose uptake, growth factor signaling, tumor invasion, and metastasis (3). Under normal oxygen tensions, HIF-1α is hydroxylated by prolyl hydroxylases, which require oxygen, ferrous ion, and 2-oxoglutarate for activity (4, 5). Once hydroxylated, HIF-1α binds rapidly to pVHL, a negative regulator of HIF-1 that directs it for proteasomal degradation through its E3 ubiquitin ligase activity (6, 7). Deprivation of oxygen by exposing cells to hypoxia prevents hydroxylase activity and stabilizes HIF-1α protein. Transition metals such as cobaltous ions that compete with ferrous ions can also inhibit hydroxylation of HIF-1α and, therefore, induce elevated HIF-1α levels under normoxia. Histologic analyses have shown that an increased level of HIF-1α protein expression is positively associated with many solid tumor types (8). Although there are conflicting findings (9–13), the majority of clinical data support a positive link between increased patient mortality and elevated HIF-1α levels (14–19). In tumor xenograft models, decreased HIF-1 activity is usually associated with a slower growing and less angiogenic tumor phenotype (20–24). Inhibition of the HIF-1

Note: C. Tan designed the experiments and did the library screening. C. Tan and H. Zhang did Western blotting analyses. R.G. de Noronha, A.J. Roecker, M.M. Pereira, and K.C. Nicolaou took part in generating the combinatorial library and resynthesizing 103D5R. Q. Teng did nuclear magnetic resonance analyses on the library and resynthesized compounds. B. Pyrzynska and Z. Zhang did Northern blotting analyses; J.J. Olson supervised Z. Zhang. F. Khwaja did the two-dimensional electrophoretic analyses. A.C. Nicholson made the LN229-HRE-AP cells. W. Zhou and P. Giannakakou helped with setting up the screening assay. E.G. Van Meir conceived the project, and C. Tan and E.G. Van Meir discussed the experimental design, analyzed and interpreted the results, and wrote the article.

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pathway may, therefore, represent an important target for antitumor intervention with pharmacologic agents.

Small-molecule inhibitors of the HIF-1 pathway are expected to attenuate hypoxia-inducible gene expression and thus retard tumor growth. Several known anticancer compounds used or tested in the clinic have been shown to have pleiotropic effects, including inhibition of HIF-1α. These include the microtubule destabilizer 2-methoxyestradiol (25), the HSP90 inhibitor 17-allyl-amino-geldanamycin (26), thioredoxin inhibitors 1-methylpropyl-2-imidazolyl disulfide (27), the topoisomerase inhibitors camptothecin and topotecan (28), and the mammalian target of rapamycin (mTOR) kinase inhibitor CCI-779 (29). Recently, chetomin, a small natural product binding to the CH1 domain of p300, a cofactor for several transcription factors including HIF, was shown to attenuate hypoxia-inducible transcription and reduce tumor growth (24). Whereas encouraging, further HIF pathway inhibitors need to be developed as chetomin induced coagulative necrosis, anemia, and leukocytosis in mice.

To identify novel HIF-1 pathway inhibitors, we screened a natural product-like combinatorial library, as >60% of anticancer drugs discovered in the past two decades are derived from natural products (30). In this paper, we describe the discovery of 103D5R, a novel natural product-like small-molecule inhibitor of the HIF-1 pathway. 103D5R rapidly down-regulates HIF-1α by decreasing its protein synthesis without affecting mRNA levels or protein degradation. Importantly, this compound prevents the transcription of HIF target genes such as vascular endothelial growth factor (*VEGF*) and glucose transporter-1 (*Glut-1*), which are essential for tumor growth.

Materials and Methods

Cell Lines. Human glioblastoma LN229 cells (31), human breast cancer MDA-MB-468 cells, and human prostate cancer PC-3 and DU-145 cells were maintained in DMEM (Mediatech, Herndon, VA) containing 1g/L glucose, supplemented with 10% fetal bovine serum, sodium pyruvate (1 mmol/L), nonessential amino acids, penicillin (50 IU/mL), and streptomycin (50 μg/mL). Cells were cultured at 37°C in a humidified atmosphere containing 5% CO_2 and 21% O_2 in air (called normoxia). For hypoxia exposure, cells were placed in a sealed modular incubator chamber (Billups-Rothenberg, Del Mar, CA) flushed with a mixture of gas consisting of 1% O_2, 5% CO_2, and 94% N_2.

Generation of LN229-HRE-AP Cell Line. The pBI-GL-HRE V6R plasmid (32), which contains six copies of HREs derived from the human *VEGF* gene, was used to generate a plasmid (pACN188) containing an alkaline phosphatase gene under the V6R HRE promoter. LN229 cells were cotransfected with the resulting plasmid and pcDNA3 vector carrying a neomycin-resistant gene. Stable clones were selected by 1,200 μg/mL G418 (Invitrogen, Carlsbad, CA) and further tested for hypoxia-inducible alkaline phosphatase expression. The cells (4×10^4 per well) were plated in 96-well plates and incubated under hypoxia or normoxia for 24 hours. Cells were washed with PBS and the buffer was then depleted. Fifty milliliters *p*-nitrophenyl phosphate (1 mg/mL, Sigma, St. Louis, MO) were added to each well and the plates were incubated at 37°C for 30 minutes. The plates were read for absorbance at 405 nm using a spectrophotometer. The clone (LN229-HRE-AP #16) with the highest expression ratio of alkaline phosphatase enzymatic activity under hypoxia versus normoxia was chosen to screen library compounds.

Screening of 10,000-Membered Natural Product-like Combinatorial Library. The library compounds were kept in DMSO in 96-well plates and stored at −20°C. LN229-HRE-AP cells (4×10^4 per well) were seeded in 96-well plates. The compounds were diluted and distributed to each plate by a BioMek 2000 workstation (Beckman Coulter, Fullerton, CA). The cells were incubated under hypoxia for 24 hours. The expression of alkaline phosphatase was measured as described above.

Cell Proliferation Assay and Long-term Clonogenicity Assay. For cell proliferation assay, LN229 cells were plated in 12-well plates (10^5 cells per well). 103D5R was added to cells at concentrations of 20 to 80 μmol/L. Cells were incubated under normoxia or hypoxia at 37°C for 96 hours. Cells were trypsinized and stained with trypan blue solution (Sigma), viable cells were then counted with a hemacytometer. For long-term clonogenicity assay, LN229 cells (500 per flask) in single cell suspension were seeded in T50 flask. Cells were incubated under normoxia at 37°C for 21 days in the presence (50 μmol/L) or absence of 103D5R. Cells were fixed with 1% glutaraldehyde and stained with 0.1% crystal violet solution.

Western Blot Analysis. Whole-cell extracts were obtained by lysing cells in radioimmunoprecipitation assay buffer [50 mmol/L Tris-HCl (pH 7.5), 150 mmol/L NaCl, 2 mmol/L EDTA, 1% NP40, 1 mmol/L phenylmethylsulfonyl fluoride] supplemented with the protease inhibitor cocktail (Roche Diagnostics, Indianapolis, IN). The proteins (50−75 μg) were resolved on 7.5% or 10% Tris-HCl gel (Bio-Rad, Richmond, CA) and transferred onto nitrocellulose membrane. The membranes were immunoblotted using anti-HIF-1α antibody (1:600 dilution, BD Biosciences, San Diego, CA), anti-HIF-1β antibody (1:1,000 dilution, Novus Biologicals, Littleton, CO), anti-IκBα antibody (1:1,000 dilution, Santa Cruz Biotechnologies, Santa Cruz, CA), and anti-β-actin antibody (1:1,000 dilution, Santa Cruz Biotechnologies). Antibodies specific for phosphorylated (Ser473, 1:500 dilution) or total Akt (1:1,000 dilution), phosphorylated (Thr202/Tyr204, 1:1,000 dilution) or total Erk1/2 (1:1,000 dilution), phosphorylated (Thr183/Tyr185, 1:500) or total stress-activated protein kinase/c-jun-NH_2-kinase (SAPK/JNK, 1:1,000), phosphorylated (Thr180/Tyr182, 1:500) or total p38 mitogen-activated protein kinase (MAPK, 1:1000) were purchased from Cell Signaling Technology, Beverly, MA. Antibodies specific for phosphorylated (Thr412, 1:500 dilution) or total p70 S6 kinase (1:500 dilution) were purchased from Upstate, (Lake Placid), NY. Immunodetection was done using the corresponding secondary horseradish peroxide–conjugated antibodies. Horseradish peroxide activity was detected using enhanced chemiluminescence (Pierce, Rockford, IL).

Northern Blot Analysis. Total RNA was isolated using Trizol (Invitrogen) according to the manufacturer's protocol. A pair of primers was used for each gene of interest to generate probes for Northern blot analysis by reverse transcription-PCR amplification. The sense and the antisense primers were 5′-ACCCTCTTCGTCGCTTCG-3′ and 5′-GGCCATTTCTGTGTGTAAGCA-3′ (*HIF-1α*); 5′-TTTACACGTCTGCGGATCTTG-3′ and 5′-CTCGCCTTGCTGCTCTACCTC-3′ (*VEGF*); 5′-TCCACGAGCATCTTCGAGA-3′ and 5′-ATACTGGAAGCACATGCCC-3′ (*Glut-1*). RNA was resolved on 1% agarose-formaldehyde gel and transferred to nylon membranes. The hybridization was done with the reverse transcription-PCR–generated cDNA probes specific for human *HIF-1α, VEGF, Glut-1,* and *glyceraldehyde-3-phosphate dehydrogenase*. Probes were labeled with [α-^{32}P] dCTP (Amersham Biosciences, Piscataway, NY) using Prime-It II Random Primer Labeling Kit (Stratagene, Cedar Creek, TX). Hybridization was carried out overnight using the ULTRAhyb buffer (Ambion, Austin, TX) at 42°C. The membrane was washed twice with buffer A containing 2× SSC and 0.2% SDS, followed by twice with buffer B containing 0.2× SSC and 0.2% SDS at 42°C. Stripping of the membrane before hybridization with another probe was done in microwave for 3 minutes with stripping buffer containing 1 mmol/L Tris (pH 8.0), 1 mmol/L EDTA, and 0.1% SDS.

Results

Identification of 103D5R as a HIF-1 Pathway Inhibitor From a Cell-Based Screening Assay. To identify novel small molecules targeting the HIF-1 pathway, we generated a human glioma cell line LN229-HRE-AP, which stably expresses a hypoxia-responsive alkaline phosphatase reporter gene. These cells displayed a 10-fold higher alkaline phosphatase activity under hypoxia compared with normoxia. We used these cells to screen for inhibitors acting on any steps involved in the transactivation

of HREs. Such inhibitors could act, for example, by decreasing HIF-1α expression or activity, decreasing nuclear localization of HIF-1α, or decreasing binding between HIF-1α and coactivators and/or HRE sequence. We screened a combinatorial library containing 10,000 natural product-like compounds. The library was synthesized on the principle of *privileged structures*, a term originally introduced to describe structural motifs capable of interacting with a variety of unrelated molecular targets (33). The 2,2-dimethylbenzopyran motif was chosen as a preferential synthetic scaffold for drug design because it is present in >4,000 natural products, and it renders sufficient lipophilicity to ensure good cell membrane penetration and will generate compounds on average with molecular weights of <500 Da (34). The screening of this library in LN229-HRE-AP cells led to the discovery of 103D5R (Fig. 1*A*). To confirm the anti-HIF-1 activity, 103D5R was resynthesized based on the schemes described previously (34), and the purity of the compound was >95% according to MS and ^{1}H nuclear magnetic resonance (data not shown). Following 24 hours of treatment under hypoxia, 103D5R displayed an EC_{50} of 35 ± 6 μmol/L against hypoxia-induced alkaline phosphatase enzymatic reporter activity (Fig. 1*B*). The effect of 103D5R on cell proliferation under normoxia was tested in LN229 cells. It had an IC_{50} of 26 ± 4 μmol/L following 96 hours of incubation (Fig. 1*C*). Up to 80 μmol/L, 103D5R had a cytostatic effect on cells. Similar antiproliferative activity of 103D5R was also observed in LN229 cells under hypoxia (data not shown). In these cells following 96 hours of treatment under normoxia or hypoxia, 103D5R (50 μmol/L) did not induce apoptosis because no cleavage of PARP or DNA fragmentation factor 45 was observed (data not shown). The cytostatic effect of 103D5R was further shown in a clonogenicity assay under normoxia for 21 days. 103D5R (50 μmol/L) completely blocked the colony formation, indicating its inhibitory effect on long-term tumor cell growth (Fig. 1*D*).

103D5R Decreases HIF-1α Protein Levels in a Dose- and Time-Dependent Manner. To explore the underlying mechanism of 103D5R activity, we investigated its effect on total HIF-1α protein levels. In LN229 cells, HIF-1α protein is undetectable under normoxia, whereas it is stabilized under hypoxia or in the presence of $CoCl_2$ and becomes readily detectable by Western blotting. Following 24 hours of treatment, 103D5R exerted dose-dependent inhibition of HIF-1α protein levels induced by hypoxia or $CoCl_2$ in these cells, with complete abrogation at 50 μmol/L (Fig. 2*A*). In contrast to the robust decrease in HIF-1α levels, 103D5R had almost no effect on HIF-1β levels. The effect of 103D5R on HIF-2α could not be evaluated due to the undetectable level of the protein in LN229 cells. Another protein, IκBα, which is ubiquitinated by a member of the E3 ligase family and subject to rapid proteosomal degradation (35), remained also largely unaffected by 103D5R treatment, except under the combined stress of $CoCl_2$ (300 μmol/L) and 103D5R (50 μmol/L). 103D5R had no effect on β-actin levels either.

The activity of 103D5R was not restricted to human glioma LN229 cells as it was also observed in human cancer cell lines derived from different tissue types, including breast (MDA-MB-468) and prostate (DU-145 and PC-3; Fig. 2*B*). Furthermore, the inhibition on HIF-1α of 103D5R did not depend on oxygen tension. Both prostate cancer cell lines exhibit constitutive HIF-1α expression under normoxia, and 103D5R was equally able to abrogate HIF-1α to undetectable levels. In contrast to LN229 cells, some of these cells showed a decrease in HIF-1β and, to a minor extent, in IκBα, whereas β-actin remained unaffected. The reason

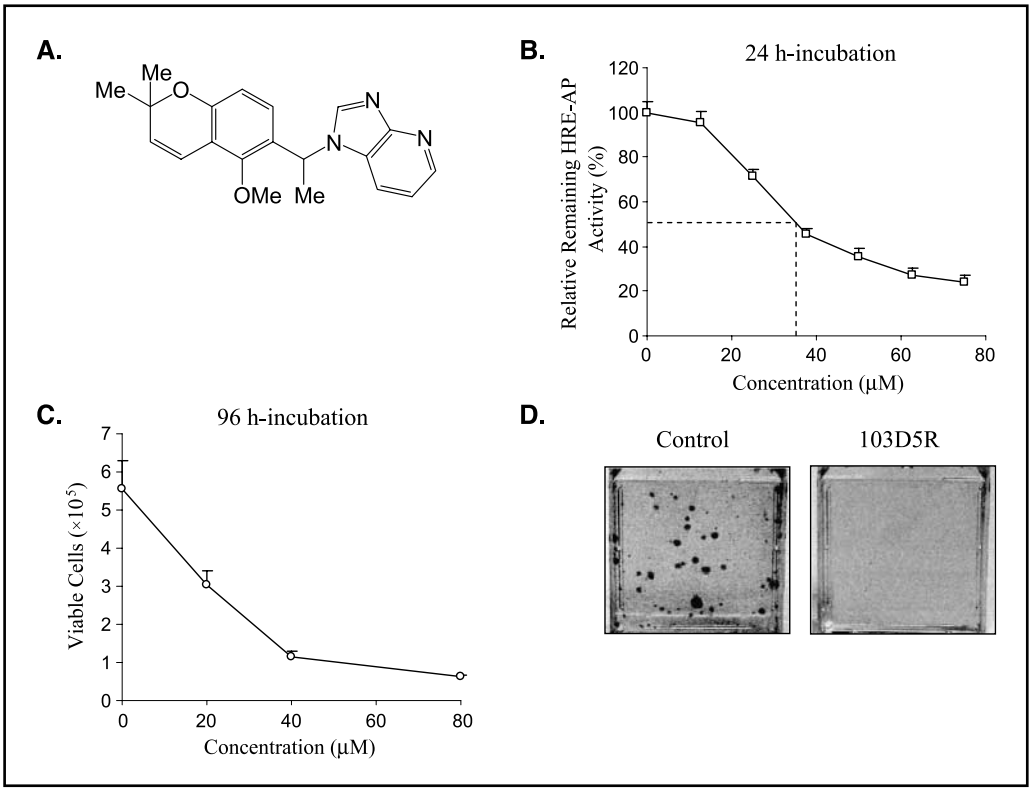

Figure 1. *A,* chemical structure of 103D5R. *B,* 103D5R inhibits HRE-mediated alkaline phosphatase (*AP*) expression under hypoxia. LN229-HRE-AP #16 cells were incubated under hypoxia in the absence or presence of 103D5R for 24 hours. The relative remaining AP activity was calculated as the ratios of AP levels in cells treated with 103D5 versus untreated cells. *Points,* means of representative data obtained from five independent experiments (n = 6); *bars,* ± SD. *C,* 103D5R inhibits cell proliferation. LN229 cells (10^5) were incubated under normoxia in the absence or presence of increasing concentration of 103D5R (20-80 μmol/L) for 96 hours. *Points,* means of representative data obtained from three independent experiments (n = 6); *bars,* ± SD. *D,* 103D5R has inhibitory effect on clonogenicity *in vitro*. LN229 cells (500 per flask) were seeded in T50 flasks and incubated under normoxia in the absence or presence of 103D5R (50 μmol/L) for 21 days. Representative data obtained from two independent experiments.

for these differences in susceptibility of HIF-1β to 103D5R remains to be explored, but one possibility is that the dimerization with HIF-1α might stabilize HIF-1β in certain cells.

The action of 103D5R was not only dose dependent, it also depended on the duration of the treatment. In LN229 cells exposed to hypoxia or $CoCl_2$, partial inhibition on HIF-1α was observed as early as 4 or 8 hours, respectively, following treatment with 50 μmol/L 103D5R, whereas complete abrogation was obtained by 24 hours (Fig. 2*C*). Again the levels of HIF-1β, IκBα, and β-actin were largely unchanged.

103D5R Decreases HIF-1α Protein Synthesis Rate but Does Not Affect HIF-1α Half-life. To further investigate the mechanism by which 103D5R inhibits HIF-1α levels, we examined whether it was the result of increased degradation or decreased synthesis of HIF-1α protein and mRNA. To specifically determine the effect of 103D5R on HIF-1α protein stability, the protein translation inhibitor cycloheximide was used to prevent *de novo* HIF-1α protein synthesis. We first induced HIF-1α accumulation by exposing the cells to 1% O_2 for 4 hours and added cycloheximide alone or in conjunction with 103D5R. In the presence of cycloheximide, HIF-1α levels declined rapidly as expected, showing a half-life of <20 minutes in LN229 cells. 103D5R did not modify the degradation rate of HIF-1α (Fig. 3*A*, compare *lanes 3-5* with *lanes 6-8*). Similar results were obtained when HIF-1α levels were induced under normoxia by addition of 300 μmol/L $CoCl_2$ for 4 hours (compare *lanes 10-12* with *lanes 13-15*). Under cobalt, the half-life of HIF-1α was around 1.5 hours in both the 103D5R-untreated and 103D5R-treated cells. The results of these experiments suggest that 103D5R does not promote HIF-1α degradation either under hypoxia or in the presence of $CoCl_2$ (Fig. 3*B*).

To specifically investigate whether 103D5R modulates HIF-1α protein synthesis, we used the proteasome inhibitor MG-132 to prevent HIF-1α degradation. This allowed us to observe HIF-1α stabilization and accumulation following *de novo* protein synthesis. Because of rapid proteosomal destruction and undetectable HIF-1α levels in normoxic cells, the accumulation rate of HIF-1α via proteosomal inhibition reflects the synthesis rate of the protein (36). As shown in Fig. 3*C* and *D*, HIF-1α rapidly accumulated over a period of 4 hours in the presence of MG-132 under both normoxia and hypoxia, as well as in the presence of $CoCl_2$. In contrast, cotreatment with 103D5R and MG-132 resulted in a much slower rate of HIF-1α accumulation (compare *lanes 3-5* with *lanes 6-8* under normoxia, *lanes 9-11* with *lanes 12-14* under hypoxia, and *lanes 15-17* with *lanes 18-20* for $CoCl_2$). Quantification by densitometry showed 103D5R treatment for 4 hours under normoxia or hypoxia led to a ~72% reduction in newly synthesized HIF-1α protein levels, as compared with untreated cells (Fig. 3*D*). Similar results were obtained in the presence of $CoCl_2$. No effects were observed on HIF-1β, IκBα, and β-actin. These results indicate that HIF-1α protein synthesis in LN229 cells is markedly impaired in the presence of 103D5R.

To determine whether HIF-1α synthesis inhibition by 103D5R was a downstream effect from decreased *HIF-1*α gene transcription or HIF-1α mRNA stability, we analyzed steady-state HIF-1α mRNA levels by Northern blotting. 103D5R did not change HIF-1α mRNA steady state levels under either normoxia or hypoxia during 4 to 24 hours of treatment (Fig. 5). This suggests that 103D5R-mediated decrease of HIF-1α synthesis is due to down-regulation of HIF-1α mRNA translation.

103D5R Decreases Phosphorylation of Akt, SAPK/JNK, and Erk 1/2 but Has No Effect on Total Akt, SAPK/JNK, and Erk 1/2 Levels. The phosphatidylinositol 3′-kinase (PI3K)-Akt-mTOR and MAPK pathways have been implicated in the regulation of HIF-1α protein synthesis at the translational level (37, 38). To address whether 103D5R inhibited HIF-1α protein synthesis by down-regulation of the PI3K-Akt-mTOR or MAPK pathways, we tested the effect of 103D5R on phosphorylated Akt, total Akt, phosphorylated Erk1/2, total Erk1/2, phosphorylated p38 MAPK, total p38 MAPK, phosphorylated SAPK/JNK, total SAPK/JNK, phospho-p70 S6 kinase, or total p70 S6 kinase levels. As shown in Fig. 4, 103D5R had no inhibitory effect on total Akt, total Erk1/2, total JNK, and

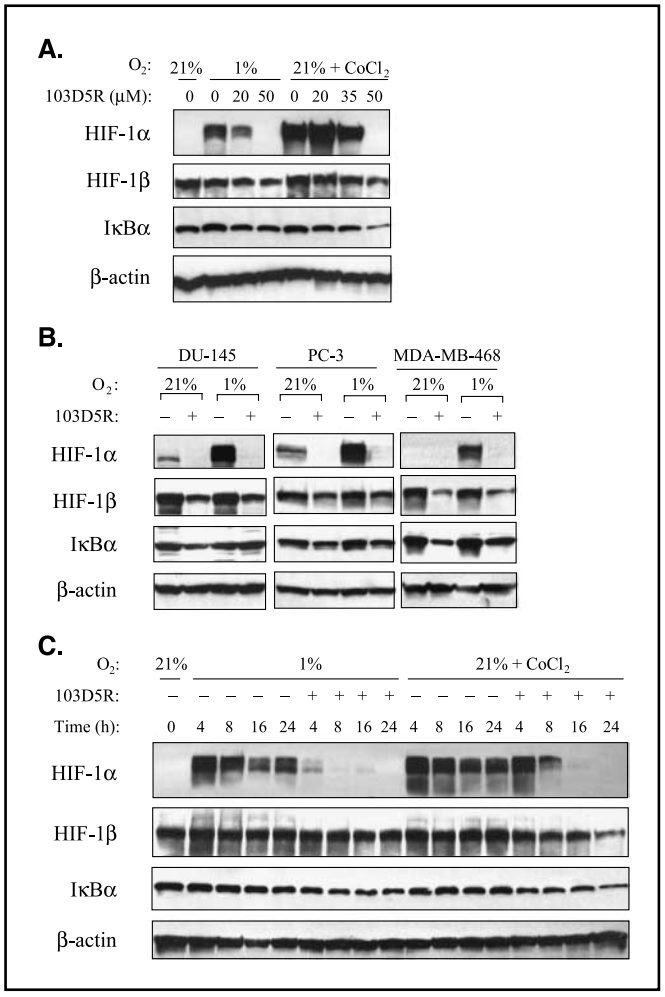

Figure 2. *A,* 103D5R inhibits HIF-1α protein in a dose-dependent manner. LN229 cells were incubated under normoxia or hypoxia for 24 hours, in the absence or presence of increasing concentration of 103D5R (20-50 μmol/L). Whole-cell lysates were analyzed by Western blotting. The same blot was reprobed with additional antibodies as indicated. β-Actin level was used as a loading control. *B,* inhibition of 103D5R on HIF-1α protein is cell line independent. Human prostate cancer DU-145 cells and PC-3 cells and human breast carcinoma MDA-MB-468 cells were incubated under normoxia or hypoxia for 24 hours, in the absence or presence of 103D5R (50 μmol/L). Whole-cell lysates were analyzed by Western blotting. The same blots were reprobed with additional antibodies as indicated. β-Actin level was used as a loading control. *C,* 103D5R inhibits HIF-1α protein in a time-dependent manner. LN229 cells were incubated under normoxia or hypoxia for 4, 8, 16, or 24 hours, in the absence or presence of 103D5R (50 μmol/L). Whole-cell lysates were analyzed by Western blotting. The same blot was probed, stripped, and reprobed with antibodies as indicated. β-Actin level was used as a loading control.

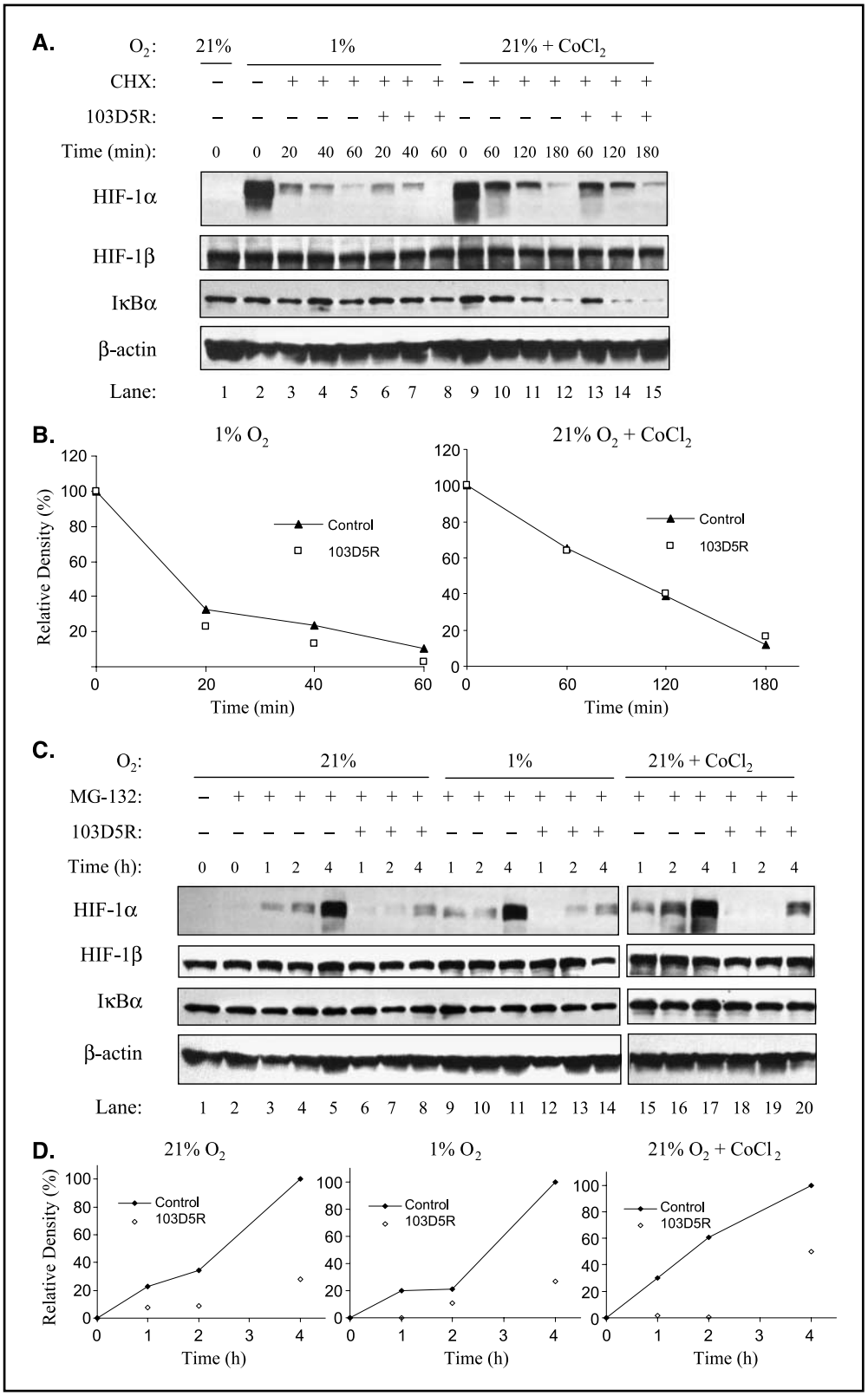

Figure 3. *A,* half-life of HIF-1α is not affected by 103D5R treatment. The cells were incubated under hypoxia or in the presence of $CoCl_2$ (300 μmol/L) for 4 hours. Cycloheximide and 103D5R were then mixed with culture media. At 20, 40, and 60 minutes (hypoxia group) or 1, 2, and 3 hours ($CoCl_2$ group) following the addition of cycloheximide, cells were harvested and whole-cell lysates were analyzed by Western blotting. The same blot was reprobed with additional antibodies as indicated. β-Actin level was used as a loading control. *B,* quantification of the HIF-1α signals by densitometry. Values were normalized to the β-actin levels and expressed as percentage relative to time 0, which was considered equal to 100%. *C,* 103D5R decreases HIF-1α protein accumulation. Proteasome inhibitor MG-132 (10 mmol/L) was added to LN229 cells for 1 hour. Cells were-treated with 103D5R (50 μmol/L) as indicated and incubated under normoxia, hypoxia or in the presence of $CoCl_2$ (300 μmol/L) for 1, 2, and 4 hours. Cells were harvested and whole-cell lysates were analyzed by Western blotting. The same blot was reprobed with additional antibodies as indicated. β-Actin level was used as a loading control. *D,* quantification of the HIF-1α signals by densitometry. Values were normalized to the β-actin levels and expressed as percentage relative to time 4 hours, which was considered equal to 100%.

total p70 S6 kinase up to 24 hours, but it clearly decreased phosphorylation of Akt, Erk1/2, and JNK at 16 and 24 hours. Phosphorylated p38 MAPK, total p38 MAPK, and phosphorylated p70 S6 kinase were not detectable in these cells (data not shown). In comparison, HIF-1α levels were already decreased at 2 hours and completely abrogated by 16 hours of 103D5R treatment. These results indicate that 103D5R inhibits phosphorylation of Akt, SAPK/JNK, and Erk1/2 after 16 to 24 hours of treatment, and therefore, may

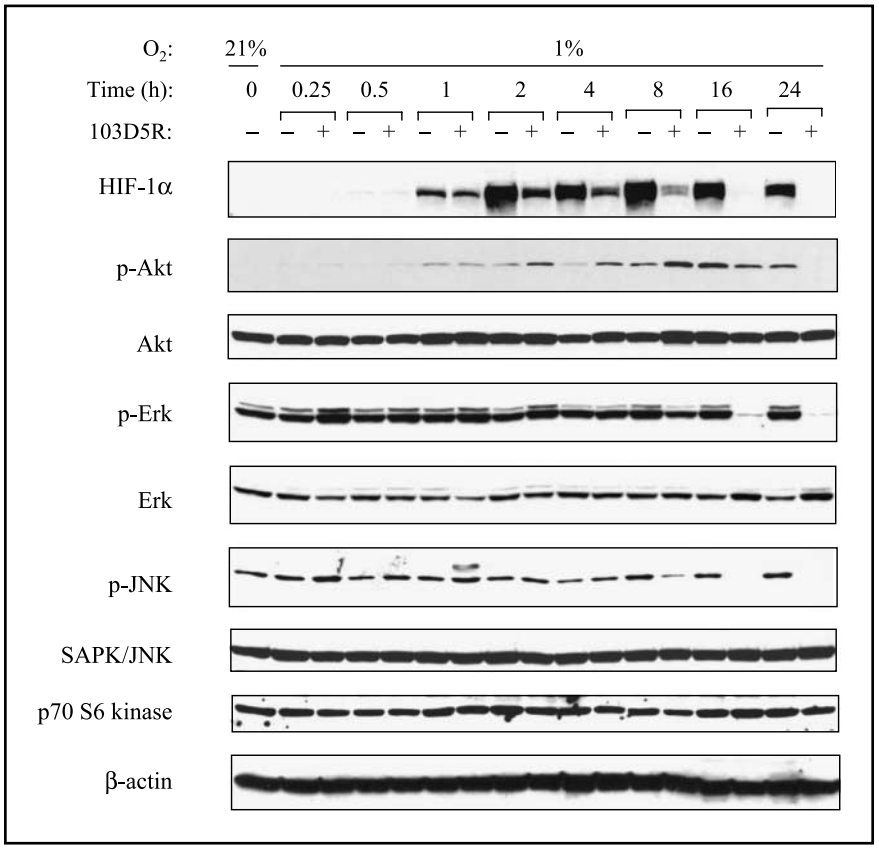

Figure 4. 103D5R inhibits the phosphorylation of Akt, Erk1/2 and SAPK/JNK but has no inhibitory effect on total levels of these proteins. LN229 cells were incubated under hypoxia for 15, 30 minutes, 1, 2, 4, 8, 16, or 24 hours in the absence or presence of 103D5R (50 μmol/L). The cells were harvested and whole-cell lysates were analyzed by Western blotting. The blots were reprobed with additional antibodies as indicated. β-Actin level was used as a loading control.

have an impact on protein translation through the mTOR-mediated pathway. Given the difference in the timing between HIF-1α inhibition and decreased phosphorylation of Akt, Erk1/2, and SAPK/JNK, further studies will have to establish whether these events are related.

103D5R Decreases Expression of HIF-1α Target Genes. To study whether the inhibition of HIF-1α would result in decreased expression of its target genes, the expression of two of the known HIF-1α target genes, *Glut-1* and *VEGF*, were analyzed. In LN229 cells, VEGF and Glut-1 mRNA levels peaked at 4 or 8 hours, respectively, after hypoxia treatment and remained elevated up to 24 hours. With 103D5R treatment, up-regulation of both HIF-1 target genes was prevented. Partial inhibition of Glut-1 mRNA up-regulation was observed as early as 4 hours following 103D5R treatment, which is consistent with the partial inhibition on HIF-1α protein by 103D5R at that time. By 24 hours, Glut-1 mRNA levels dropped to basal levels (Fig. 5). These results indicate that 103D5R is very effective in inhibiting the activation of HIF-1 target genes, which are vital to many aspects of tumor biology, including angiogenesis and metabolic adaptation for survival under anaerobic conditions.

Discussion

In this study, we identified 103D5R as a novel small molecule inhibitor of the HIF-1 pathway from screening a combinatorial library which has a 2,2-dimethylbenzopyran structural motif. We showed that 103D5R inhibited HIF-1α levels by impairing HIF-1α protein synthesis, indicating it acted as an inhibitor of HIF-1α mRNA translation. This inevitably raises the question as to the specificity of 103D5R as a HIF-1α inhibitor. To address this issue, we sampled the proteomic profiles of LN229 cells with or without 103D5R treatment under hypoxia. The two-dimensional gel electrophoresis (pH 4.5-7.0) results showed that 95% of 300 proteins detected showed no alteration in expression levels (data not shown). To further examine

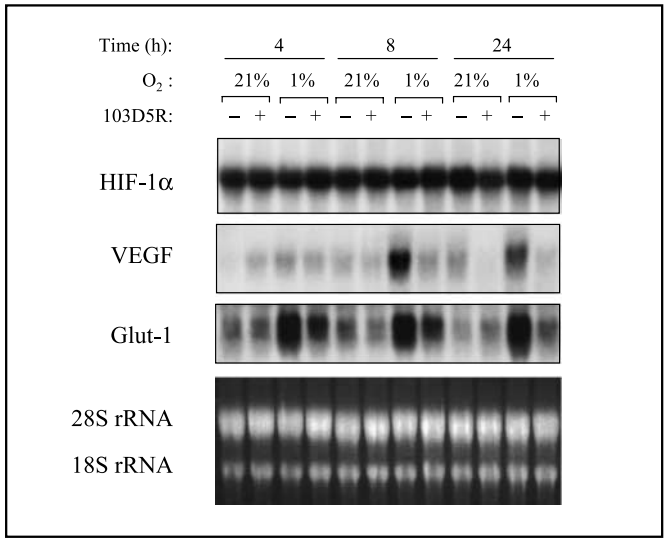

Figure 5. 103D5R does not affect HIF-1α mRNA levels but inhibits transcription of HIF-1α target genes. LN229 cells were incubated under normoxia or hypoxia for 4, 8, or 24 hours in the absence or presence of 103D5R (50 μmol/L). Total RNA was analyzed by Northern blotting. The intensity of 28S and 18S rRNA by ethidium bromide staining was used as loading control.

specific proteins involved in the HIF-1 pathway, mRNA translation pathway and a short-lived protein, we further analyzed total protein levels of HIF-1β, Akt, Erk1/2, SAPK/JNK, p38 MAPK, p70 S6 kinase, and IκBα. In contrast to HIF-1α, which was decreased the most and the earliest by 103D5R, the expression levels of these proteins were either not inhibited or inhibited to a much lesser extent, suggesting that 103D5R was not a general protein translation inhibitor. The fact that the translation of HIF-1α mRNA can be preferentially inhibited by 103D5 implies that it may be more susceptible to inhibition than that of other transcripts. One possible explanation is that HIF-1α mRNA translation may involve some specialized mechanism, which can be targeted without affecting the translation machinery employed by the majority of genes. Translation of most eukaryotic mRNAs requires the interaction between the mRNA 5′ -methylated guanosine cap with eIF4E, which is a component of the eIF4F translation initiation complex. As an alternative, 5′ -cap–independent translation recruits the translation initiation complex by an internal ribosome entry site. HIF-1α mRNA has been reported to contain an internal ribosome entry site that allows its efficient translation under both normoxia and hypoxia (39). If this holds true, further studies are justified to examine whether 103D5R may preferentially impair HIF-1α mRNA translation by interfering with internal ribosome entry site–dependent translation initiation.

In many cancers, the HIF-1 pathway is not only activated by low oxygen tension, it is also induced or amplified by a wide range of growth-promoting stimuli and oncogenic pathways. Increased HIF-1α protein synthesis via the activation of PI3K-Akt-mTOR or MAPK pathways is a common theme accounting for the up-regulation (37, 38). In this study, we have shown that 103D5R significantly decreased phosphorylation of Akt, Erk1/2, and SAPK/JNK. Further studies are warranted to determine whether inhibition of these phosphorylation events by 103D5R are related to decreased HIF-1α synthesis. Under the conditions of constitutive activation used here, it is difficult to interpret the timing of the *de novo* protein phosphorylation as it is confounded by the half-lives of preexisting phosphorylated proteins that are unaffected by 103D5R treatment. Inhibition of PI3K-Akt-mTOR and MAPK pathways is expected to have a broader impact on protein translation than HIF-1α alone. Both pathways are closely involved in the phosphorylation and activation of eIF4E, a key translation factor that mediates the initiation of mRNA translation (40). Furthermore, these signaling cascades also lead to rRNA and tRNA gene transcription (41, 42) and ribosome biogenesis through transcription of ribosomal protein genes (43). All these events are likely to slow down cell proliferation, and we observed that continuous exposure to 103D5R was cytostatic. More studies are currently ongoing to further investigate the mechanism by which 103D5R inhibits protein synthesis.

Due to the genetic activation of a number of signaling pathways in tumors that stimulate mRNA translation, it may be possible to inhibit a specific activating component of the translational machinery in tumors without affecting its function in normal cells. 103D5R was able to abolish the stabilization of HIF-1α by hypoxia and up-regulation of key HIF-1 target genes such as *VEGF* and *Glut-1* in cancer cells of different tissue origins. Given the importance of HIF-1–activated gene products in growing solid tumors, it will be important to evaluate the antitumor efficacy of 103D5R in preclinical models once its toxicity, pharmacokinetics, and pharmacodynamics have been established.

We also plan to improve the potency of 103D5R through structure-activity relationship studies. This will include building and screening a follow-up library in which the different regions of 103D5R will be refined: (1) the substitution pattern in the left-hand benzopyran ring system will be varied; (2) the right-hand heterocyclic aromatic system will be substituted by other heterocyclic rings; and (3) the alkyl group in the linker region will be replaced with other aliphatic and aromatic substituents. As a result of such systematic structure-activity relationship studies on 103D5R structure, we expect to design a new molecule which will have a more potent inhibitory activity against the HIF-1 pathway.

In summary, we have shown that 103D5R decreased HIF-1α protein levels under both normoxic and hypoxic conditions. This was associated with a decrease in HIF-1α protein synthesis and the prevention of HIF-1 target gene activation. Precisely how 103D5R inhibits HIF-1α protein synthesis and why this inhibitory effect shows specificity remain to be elucidated. A better understanding of these mechanisms will undoubtedly provide new insights into HIF biology and opportunities for pharmacologic and therapeutic intervention in HIF-1-driven tumor growth.

Acknowledgments

Received 9/7/2004; revised 11/9/2004; accepted 11/12/2004.

Grant support: NIH grants CA86335 and CA87830 (E.G. Van Meir), F32 CA101509-02 (C. Tan), and T32 NS07480 (A.C. Nicholson) and Foundation for Science and Technology (Portugal) grant SFRH/BD/5371/2001 (R.G. de Noronha).

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We thank Drs. Donald Durden, Pradip De, Hua Zhong, Haian Fu, and Yue Feng for reagents, experimental advice, and stimulating discussions, and Drs. Jin H. Song and Chunhai Hao for helping with the apoptosis assay.

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