Topotecan inhibits vascular endothelial growth factor production and angiogenic activity induced by hypoxia in human neuroblastoma by targeting hypoxia-inducible factor-1α and -2α

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Abstract

Neuroblastoma produce angiogenic peptides, and the extent of angiogenesis correlates with tumor progression and poor clinical outcome. Hence, angiogenic factor inhibition represents an important therapeutic option. One of the major drives to tumor angiogenesis is hypoxia, a decrease in oxygen tension that characterizes the tumor microenvironment. We investigated the effects of the topoisomerase I inhibitor, topotecan, on vascular endothelial growth factor (VEGF) induction by hypoxia in advanced-stage human neuroblastoma cells. Topotecan counteracted hypoxic induction of VEGF and decreased angiogenic activity of conditioned medium from hypoxic cultures *in vivo* in the chick chorioallantoic membrane. Promoter-driven reporter studies showed the role of both hypoxia-inducible factor (HIF)-1α and -2α in VEGF transcription activation by hypoxia, because (*a*) overexpression of either protein by cotransfection with expression vectors resulted in VEGF promoter transactivation, which was abrogated by mutation in the HIF-binding site, and (*b*) targeted knockdown of HIF-1α/2α by RNA interference inhibited hypoxia-stimulated VEGF transcriptional activity and protein secretion. Topotecan-inhibitory effects on VEGF induction by hypoxia were mediated through suppression of both HIF-1α and HIF-2α protein accumulation and transactivation properties, which was specific and required ongoing RNA transcription. A similar pattern of results was obtained in cells treated with the hypoxia-mimetic agent, desferrioxamine. These data provide the first evidence that topotecan is a potent inhibitor of HIF-1α and HIF-2α subunits in hypoxic neuroblastoma cells, leading to decreased VEGF expression and angiogenic activity. An important clinical implication of these findings is that therapies targeted to the HIF pathway have the potential to inhibit neuroblastoma angiogenesis and growth. [Mol Cancer Ther 2008;7(7):1974–84]

Introduction

Neuroblastoma is the most common extracranial solid tumor of children, which arises from the sympathetic nervous system (1). Neuroblastoma tumors exhibit clinical and biological heterogeneity and are characterized by genetic mutations, which correlate with patient prognosis (1–3). Survival rates for children who develop advanced-stage neuroblastoma over age 1 year is low despite the array of chemotherapeutic agents available and aggressive multimodality therapeutic protocols (2, 4), and the identification of more effective therapies remains a primary goal.

Malignant solid tumors depend on neoangiogenesis for growth and metastatization (5). Tumor angiogenesis is tightly regulated by a balance of stimulatory and inhibitory factors released by tumor and infiltrating cells and is influenced by signals of various nature (5, 6). Angiogenic factor production by neuroblastoma cells has prognostic potential, correlating with MYCN oncogene amplification, metastatic disease, and poor clinical outcome (7, 8). Hence, pharmacologic inhibition of angiogenesis has potential therapeutic implication for neuroblastoma treatment, and new antiangiogenic agents are currently being tested (8).

One of the major drives to tumor angiogenesis is hypoxia (9), a local decrease in oxygen tension ($pO_2 < 20$ mm Hg) that characterizes the tumor microenvironment (10, 11). Neuroblastoma tumors present areas of hypoxia and relapse at hypoxic sites, such as bone and bone marrow (1, 2). Recent studies documented an important role for hypoxia in neuroblastoma cell acquisition of a neural crest–like phenotype, which in the clinical setting is associated with increased aggressiveness, adverse outcome, and resistance to therapy (12–14). In particular, high expression of the hypoxia target proangiogenic cytokine, vascular endothelial growth factor (VEGF), in neuroblastoma tumors is a marker of poor prognosis (13, 15, 16).

Gene transactivation by hypoxia is mediated primarily by the hypoxia-inducible factor (HIF), a heterodimer of a

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constitutive β-subunit (HIF-1β) and an oxygen-regulated α-subunit (HIF-1α/2α). The α-subunits are post-translationally stabilized under hypoxia and translocate to the nucleus where they heterodimerize with HIF-1β, transactivating the hypoxia-responsive element (HRE) in the promoter of responsive genes (17, 18). HIF-1α/2α overexpression is associated with increased tumor angiogenesis and progression, treatment failure, and poor prognosis (9, 19–21), providing a rationale for targeting the HIF pathway as a strategy for cancer treatment (17, 22, 23). One potential candidate in this line of research is the camptothecin analogue, topotecan, a topoisomerase I inhibitor endowed with antitumor activity both in xenograft models (24) and in patients with recurrent tumors (for a review, see ref. 25), which is currently under clinical evaluation for advanced-stage, refractory neuroblastoma treatment (25, 26). Topotecan has raised considerable interest because of its ability to suppress HIF-1α expression (22), causing concomitant inhibition of HIF-1 target genes, angiogenesis, and tumor growth in a preclinical glioblastoma model (27).

HIF-1α/2α accumulation was observed in human neuroblastoma cell lines exposed to *in vitro* hypoxia and *in vivo* in the hypoxic areas of experimental tumors (28), but their relative contribution to the regulation of hypoxia target genes has not been fully clarified. Moreover, little is known on topotecan activity on hypoxia-driven genes in neuroblastoma. In this study, we used RNA interference to determine the specific roles of HIF-1α/2α in VEGF induction by hypoxia in advanced-stage MYCN-amplified human neuroblastoma cells and investigated topotecan effects on VEGF production by hypoxic cells and on their angiogenic potential. We show the contribution of both α-subunits to VEGF transcriptional activation by hypoxia and provide the first evidence that topotecan is a potent inhibitor of VEGF hypoxic induction and *in vivo* angiogenic activity of neuroblastoma cells by targeting both HIF-1α and HIF-2α. Furthermore, we show similar inhibitory effects of topotecan on HIF-1α/2α expression and activity induced under normoxia by the hypoxia-mimetic agent, desferrioxamine.

Materials and Methods

Cell Culture and Reagents

The human neuroblastoma cell lines, GI-LI-N and LAN-5, derived from metastatic, stage IV tumors and characterized by MYCN amplification (29), were purchased from the Interlab Cell Line Collection and cultured in a humidified incubator containing 20% O_2, 5% CO_2, and 75% N_2 as described (15). Hypoxic conditions (1% O_2) were achieved by culture in an anaerobic workstation incubator (BUG-BOX) flushed with a gas mixture of 1% O_2, 5% CO_2, and 94% N_2. pO_2 in culture medium was measured using a trace oxygen analyzer (Oxi 315i/set, WTW), as detailed (30).

Topotecan (GlaxoSmithKline) was dissolved in DMSO as a 2 mmol/L stock solution; desferrioxamine (Sigma) was prepared as a 25 mmol/L aqueous solution; actinomycin D (Calbiochem, VWR) was dissolved in DMSO as 1 mg/mL stock.

Determination of VEGF mRNA Expression

VEGF mRNA expression was determined by Northern blot analysis or quantitative real-time PCR on total RNA purified from single-cell suspensions by the Qiagen RNeasy Mini Kit as reported (15, 30). Quantitative real-time PCR data were normalized on the values obtained in parallel for two reference genes selected by microarray as described (30).

ELISA

Secreted VEGF was measured in cell-free conditioned medium using the Quantikine immunoassay kit from R&D Systems (Space). Absorbance was determined by a Spectrafluor Plus plate reader (TECAN) at 450 nm. Data were analyzed with the Graph Pad Prism 3 Software.

Chick Embryo Chorioallantoic Membrane Assay

Chorioallantoic membrane assay was done according to the method of Ribatti et al. (31). Briefly, fertilized white Leghorn chicken eggs (10 per group) were incubated at 37°C for 8 days. Growing chorioallantoic membrane were then overlayed with 1 mm^3 sterilized gelatin sponges (Gelfoam Upjohn) loaded with 3 μL RPMI 1640 alone or containing 1 μg recombinant VEGF (R&D Systems), respectively, used as negative and positive controls, 3 μL conditioned medium from LAN-5 cells cultured under hypoxia, in the presence or absence of 500 ng goat anti-human VEGF polyclonal antibody (R&D Systems), or conditioned medium from hypoxic LAN-5 cells treated with 500 nmol/L topotecan. Chorioallantoic membrane were examined daily and photographed *in ovo* with a stereomicroscope equipped with a Camera System MC63 (Zeiss). At day 12 of incubation, angiogenic response was evaluated macroscopically by counting the number of allantoic vessels converging radially toward the sponges.

Western Blot

Western blot analysis was done as detailed (32). Briefly, total cell lysates (100 μg) were electrophoresed on a 8% SDS-PAGE and electroblotted to Immobilon-P nitrocellulose membranes (Millipore). Immunoblotting was done with anti-HIF-1α mouse monoclonal antibody (BD Biosciences), anti-HIF-2α rabbit polyclonal antibody (Novus, Valter Occhiena), and anti-nuclear factor-κB (NF-κB; p50 and p65) rabbit polyclonal antibodies (Santa Cruz Biotechnology). Anti-HIF-1β mouse monoclonal antibody (Novus) was used as an internal loading control. Detection was carried out by enhanced chemiluminescence (Pierce) with peroxidase-conjugated goat anti-mouse or anti-rabbit antibodies (Sigma). Quantitative assessment of band intensities was carried out with the VersaDoc Image Analyzer (Bio-Rad).

RNA Interference

Small interfering RNA (siRNA) oligonucleotides targeting HIF-1α and HIF-2α were synthesized by Qiagen according to the sequences published by Sowter et al. (33). A nonsilencing siRNA (sense 5′-UUCUCCGAACGUGUCACGUdTdT-3′ and antisense 5′-ACGUGACACGUUCGGAGAAdTdT-3′) was used as a control. LAN-5 cells were transfected at ≈50% confluency with 100 nmol/L siRNA, alone or in

combination, in serum-free Opti-MEM I medium using Oligofectamine (Invitrogen), according to the manufacturer's instructions, and incubated for 24 h before treatment.

Plasmids

Reporter constructs VEGF-p7, encoding the *firefly* luciferase gene driven by the human VEGF promoter fragment from −1,005 to +379 relative to the transcription initiation site, VEGF-p11wt, containing a 47-bp fragment from the human VEGF 5′-flanking sequence (−985 to −939) encompassing the HIF-1-binding site at position −975 linked to the minimal SV40 (ΔSV40) promoter, and VEGF-p11mut, containing a 3-bp mutation in the HIF-1-binding site (34), were purchased from the American Type Culture Collection. pGL2-3x-HRE construct, encoding the luciferase gene driven by three tandem copies of the HRE from the mouse inducible nitric oxide synthase (35) upstream of the ΔSV40 promoter, pShuttle-1α and pShuttle-2α plasmids, encoding the human HIF-1α (3.4 kb) and human HIF-2α (2.8 kb) full-length cDNA driven by the human cytomegalovirus immediate-early promoter/enhancer, were kindly provided by Dr. G. Melillo (National Cancer Institute-Frederick Cancer Research and Development Center). pShuttle control vector and pGL2-control vector containing the ΔSV40 promoter/enhancer were purchased from Clontech and Promega, respectively.

Cell Transfection and Luciferase Assay

Transient transfection was done on cells at ≈50% confluency in 6 cm^2 culture dishes using LipofectAMINE and Plus reagent (Invitrogen). To correct for variations in DNA uptake, each construct (2 μg DNA/plate) was cotransfected with pRL-TK luciferase control vector (1 μg/plate) containing the *Renilla* luciferase gene under control of the HSV-TK promoter (Promega). Twenty-four hours after transfection, cells were treated as indicated, and *firefly* and *Renilla* luciferase activities were assayed as described (32). In a set of experiments, luciferase constructs were cotransfected with HIF-1α/2α siRNA as described for RNA interference, except that Lipofectamine 2000 was used in place of Oligofectamine, and luciferase assay was done.

Statistical Analysis

Data are mean ± SE of at least three independent experiments. Statistical significance of the results ($P < 0.05$) was calculated by the Student's t test.

Results

Topotecan Inhibits Hypoxia-Induced VEGF Production by Neuroblastoma Cells

We reported that hypoxia induces VEGF production by advanced-stage human neuroblastoma cells (15). To investigate whether topotecan affected the response to hypoxia, the LAN-5 cell line was incubated under normoxic or hypoxic conditions in the presence or absence of increasing concentrations of topotecan, and VEGF mRNA levels were evaluated by Northern blot (Fig. 1A). VEGF transcript levels were significantly up-regulated on exposure to 1% O_2 for 6 h, further increasing after 18 h. mRNA induction was inhibited by ≈50% already after 6 h culture with 100 nmol/L topotecan and abrogated after 18 h exposure to 500 nmol/L. Under these experimental conditions, expression of rRNA 28S and 18S (Fig. 1A) or cell viability

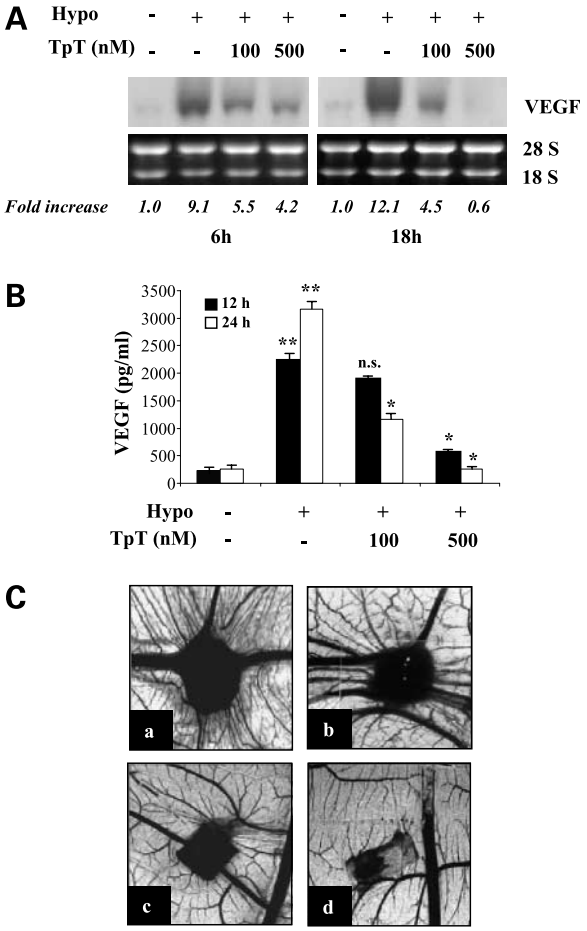

Figure 1. Dose- and time-dependent inhibition of hypoxia-induced VEGF production and angiogenic activity by topotecan in LAN-5 cells. LAN-5 cells were treated with 100 or 500 nmol/L topotecan for the indicated length of time under normoxic (-) or hypoxic (+) conditions. **A,** total RNA was isolated and analyzed for VEGF mRNA expression by Northern blot. 28S/18S rRNA is shown as a loading control. Representative of five experiments. VEGF fold increases, determined by densitometric analysis of the band intensity and normalized to the corresponding levels of 18S rRNA, are indicated relative to normoxic values (defined as equal to 1). **B,** conditioned medium was harvested and assayed for the content of secreted VEGF by ELISA. Samples were run in triplicate for each condition. Individual histograms represent mean ± SE (pg/1 × 10^6 cells/mL) of five independent experiments. **, $P < 0.01$ versus respective normoxic levels; *, $P < 0.01$ versus respective hypoxic levels. **C,** angiogenic activity of conditioned medium from cells cultured for 24 h under hypoxia in the presence or absence of 500 nmol/L topotecan was evaluated by chorioallantoic membrane assay. Chorioallantoic membrane of 8-day-old chick embryos were incubated for 4 d with gelatin sponges adsorbed with conditioned medium from hypoxic LAN-5 cells (*a*), medium containing 1 μg VEGF (*b*), conditioned medium from hypoxic LAN-5 cells + 500 ng anti-VEGF antibody (*c*), and conditioned medium from topotecan-treated hypoxic LAN-5 cells (*d*) and then photographed. Bar, 0.3 μm.

(data not shown) was not affected, with a maximum of 15% cell death caused by 500 nmol/L topotecan after 18 h treatment as assessed by the trypan blue dye exclusion test.

VEGF mRNA inhibition by topotecan was paralleled by decreased protein secretion (Fig. 1B). A 9.7-fold and 12.3-fold up-regulation of secreted VEGF was observed after 12 and 24 h culture under hypoxia. Treatment with 100 nmol/L topotecan for 24 h reduced hypoxia stimulatory effects by ≈70%, whereas >80% inhibition was achieved after 12 h and complete abrogation after 24 h culture with 500 nmol/L. These data show that topotecan is a potent inhibitor of VEGF production induced by hypoxia in neuroblastoma.

Inhibition by Topotecan of Hypoxic Neuroblastoma Cell Angiogenic Activity *In vivo*

Next, we investigated the effects of topotecan on the *in vivo* angiogenic activity of hypoxic LAN-5 cells by chorioallantoic membrane assay (Fig. 1C). Conditioned medium from hypoxic cultures stimulated a pronounced angiogenic response as shown by the presence of numerous allantoic vessels converging radially toward the implant in a ''spoked wheel'' pattern (32 ± 4; *a*), which was higher than that induced by conditioned medium from normoxic cells (data not shown) and comparable with that induced by recombinant VEGF used as a positive control (30 ± 5; *b*). A significant decrease in the number of radiating vessels ($P < 0.001$) was observed when the sponges were loaded

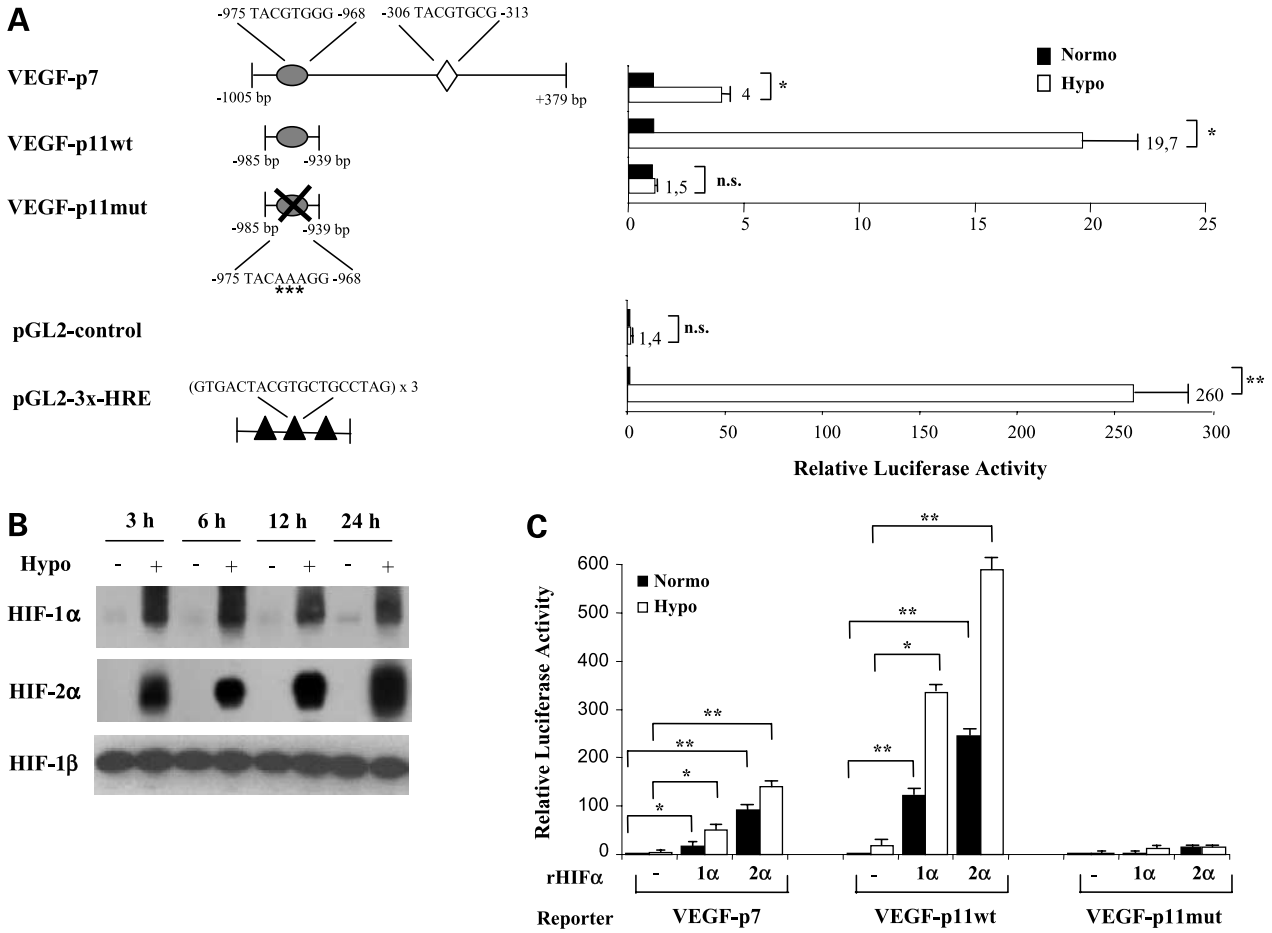

Figure 2. HIF-1α- and HIF-2α-mediated VEGF promoter transactivation in neuroblastoma cells. **A,** requirement of the −975 HIF-binding site for VEGF promoter transactivation by hypoxia. LAN-5 cell line was transiently transfected with the indicated *firefly* luciferase reporter constructs, along with the pRL-TK *Renilla* luciferase control vector, and luciferase activity was measured after exposure to normoxia (*Normo*) or hypoxia (*Hypo*) for 18 h. *Left,* schematic representation of the VEGF promoter fragments. Sequences of the wild-type or mutated HIF-1-binding sites are indicated (*asterisks,* substituted base pairs). *Right,* relative *firefly* luciferase activity of hypoxic (*open columns*) versus normoxic (*solid columns*) cells (defined as equal to 1) after normalization to the *Renilla* luciferase control vector. Mean ± SE of five independent experiments. Fold increase values are indicated. *, $P < 0.01$; **, $P < 0.001$. **B,** kinetics of HIF-1α and HIF-2α accumulation in response to hypoxia. Total protein lysates (100 μg) from LAN-5 cells cultured under normoxic or hypoxic conditions for the indicated time points were analyzed for HIF-1α and HIF-2α expression by Western blot. HIF-1β was evaluated in parallel as a loading control. Representative blot of three experiments. **C,** cotransfection of VEGF promoter-reporter plasmids and rHIF-α expression vectors. LAN-5 cells were transiently cotransfected with the indicated reporter constructs and with either the pShuttle control vector (-), pShuttle-1α (*1α*), or pShuttle-2α (*2α*) expression plasmids, respectively, containing the recombinant human HIF-1α or the recombinant human HIF-2α full-length cDNA. Luciferase activity was measured as in **A** and is presented relative to normoxic cells cotransfected with control pShuttle (arbitrarily defined as 1) for each reporter plasmid. Mean ± SE of three independent experiments. *, $P < 0.01$; **, $P < 0.001$.

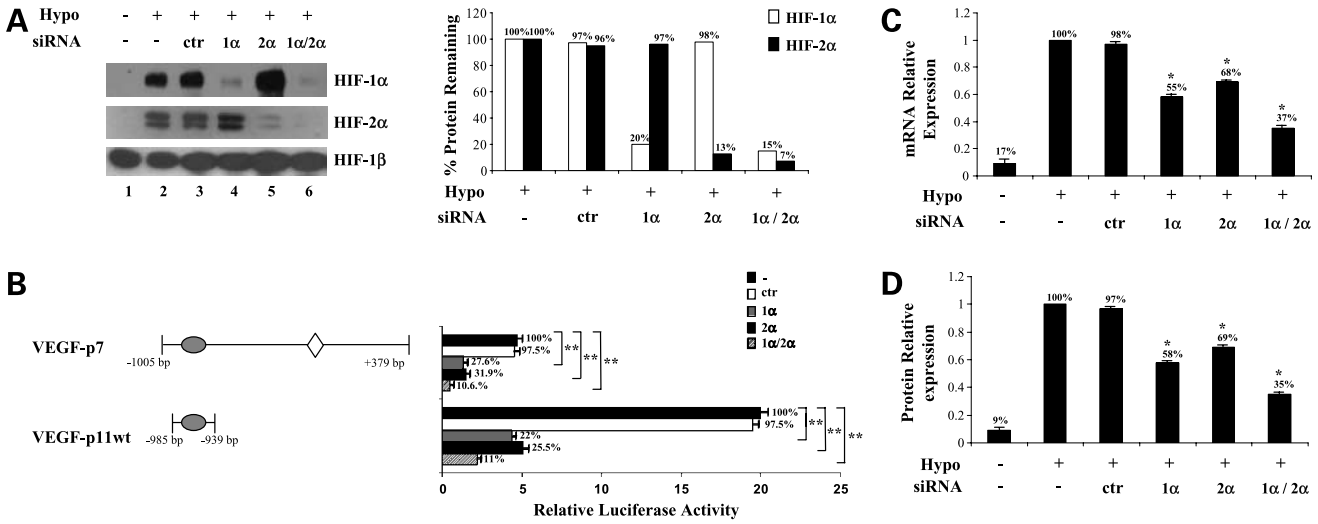

Figure 3. Inhibition of hypoxic VEGF induction by HIF-1α and HIF-2α knockdown. LAN-5 cells were transfected with 100 nmol/L siRNA to HIF-1α, HIF-2α, HIF-1α + HIF-2α, or control (*Ctr*), either alone (**A**, **C**, and **D**) or with the indicated reporter constructs (**B**), and then exposed to normoxia or hypoxia for 18 h. **A,** suppression of HIFα protein expression. HIFα protein levels were evaluated by immunoblotting as described in Fig. 2B legend. *Left,* representative autoradiograph. The extent of HIF-1α and HIF-2α knockdown was determined by PhosphoImager analysis of the blot and normalization to the corresponding HIF-1β levels. *Right,* percentage of protein remaining after silencing, taking as 100% the expression levels of untransfected hypoxic cells. **B,** inhibition of VEGF promoter transactivation. Luciferase activity was measured in the LAN-5 cell line transiently cotransfected with VEGF-p7 or VEGF-p11wt constructs and siRNA. Relative luciferase activity of hypoxic versus normoxic cells (arbitrarily defined as 1) is reported as in Fig. 2A. Mean ± SE of three independent experiments. The number associated with each column indicates the percentage of luciferase activity remaining after siRNA transfection relative to untransfected hypoxic cells (arbitrarily defined as 100%; *solid columns*). **, $P < 0.01$. **C,** quantification of VEGF mRNA down-regulation. Total RNA was reverse transcribed, and VEGF transcript levels were quantified by quantitative real-time PCR. Data are expressed as relative mRNA levels, taking as 100% the expression amounts of untransfected hypoxic cells. Mean ± SE of three independent experiments. The percentage of remaining mRNA is indicated. *, $P < 0.05$. **D,** inhibition of VEGF protein secretion. Supernatants were harvested after 24 h and assayed for secreted VEGF by ELISA. Results are expressed as the relative amounts of protein remaining after siRNA transfection relative to untransfected hypoxic cells (arbitrarily defined as 100%). Mean of three experiments. *, $P < 0.05$.

with conditioned medium from hypoxic cells supplemented with a neutralizing anti-VEGF antibody (14 ± 3; *c*), suggesting an important role for VEGF in mediating their angiogenic activity. Interestingly, treatment with topotecan (*d*) reduced to a similar extent ($P < 0.001$) the number of radiating vessels around the implant (13 ± 2). These results suggest that topotecan-dependent inhibition of VEGF production results in a significant decrease of the capacity of hypoxic neuroblastoma cells to stimulate angiogenesis *in vivo*.

HIF-1α and HIF-2α Activate VEGF Gene Transcription in Neuroblastoma Cells

VEGF induction by hypoxia occurs through both gene transcription activation and mRNA stabilization (34, 36). To determine whether hypoxia effects in neuroblastoma were transcriptionally regulated, we transfected VEGF promoter-reporter constructs into LAN-5 cells and assayed luciferase activity under hypoxia (Fig. 2A). Reporter gene expression driven by the pGL2-3x-HRE construct, used as a positive control, was significantly increased in hypoxic relative to normoxic cells. The VEGF-p7 construct, encoding the luciferase gene under control of a 1-kb fragment of the human VEGF promoter (34), showed low basal activity, and exposure to hypoxia resulted in a median 4-fold luciferase increase above the baseline in five independent experiments, indicating that VEGF 5′-flanking sequences can mediate transcriptional response to hypoxia in neuroblastoma cells. Luciferase activity was increased by 20-fold in hypoxic cells transfected with the VEGF-p11wt construct, which contains a 47-bp fragment of the human VEGF promoter encompassing the HIF-1-binding site at nucleotide −975 (34), showing that this sequence was sufficient to drive reporter gene expression in the contest of a heterologous promoter otherwise minimally responsive to hypoxia (pGL2-control). A 3-bp mutation of the HIF-binding site (VEGF-p11mut construct) completely abrogated reporter gene inducibility by hypoxia, indicating its requirement for VEGF promoter transactivation by hypoxia.

LAN-5 cells expressed constitutively almost undetectable levels of HIF-1α and HIF-2α proteins, which were significantly up-regulated by hypoxia, although with a different kinetics (Fig. 2B). HIF-1α expression was rapidly induced to peak levels within 3 h culture, starting to decline at 12 h, but persisting at high levels up to 24 h. In contrast, HIF-2α protein accumulation increased over time reaching peak levels at 24 h. HIF-1β levels were substantially unaffected by hypoxia at all the time points analyzed. To assess the relative contribution of HIF-1α versus HIF-2α in VEGF transcription activation by hypoxia, we did cotransfection experiments with VEGF reporter constructs and HIF-1α/2α-encoding vectors (Fig. 2C). Recombinant HIF (rHIF)-1α overexpression significantly increased luciferase

activity under normoxia in both the context of the native (VEGF-p7; *lane 3*) or the heterologous (VEGF-p11wt; *lane 9*) promoters (16- and 122-fold relative to control vector-transfected cells; *lanes 1* and *7*) and the magnitude of response exceeded that induced by hypoxia alone by 4.4-fold (*lane 2*) and 6.8-fold (*lane 8*), respectively. Exposure to 1% O_2 further enhanced rHIF-1α-driven promoter transactivation by 3.2- and 2.7-fold in VEGF-p7-transfected (*lane 4*) and VEGF-p11wt-transfected (*lane 10*) cells, respectively, compared with normoxic counterparts (*lanes 3* and *9*) and by 14- and 18.6-fold respect to hypoxic cells without rHIF-1α (*lanes 2* and *8*), suggesting a synergistic activity of rHIF-1α and hypoxia. A similar response was observed on rHIF-2α overexpression, which significantly increased VEGF-p7-driven (*lane 5*) and VEGF-p11wt-driven (*lane 11*) luciferase expression under normoxic conditions (92- and 245-fold relative to control vector-cotransfected cells; *lanes 1* and *7*), with the extent of induction exceeding that of hypoxia alone by 25.5-fold (*lane 5* versus *lane 2*) and 13.6-fold (*lane 11* versus *lane 8*), respectively. Hypoxia further increased rHIF-2α inducibility by 1.5- and 2.4-fold in VEGF-p7-transfected (*lane 6* versus *lane 5*) and VEGF-p11wt-transfected (*lane 12* versus *lane 11*) cells, respectively, compared with normoxic counterparts, and by 39- and 32.7-fold respect to hypoxic cells without rHIF-2α (*lanes 2* and *8*). HIF-1-binding site mutation abrogated rHIF-1α and rHIF-2α-driven transcriptional activity both under normoxia and hypoxia. We conclude that VEGF transcription in neuroblastoma cells can be significantly induced by overexpression of either α-subunit via the HIF-1-binding site.

HIF-1α and HIF-2α Are Required for VEGF Production by Hypoxic Neuroblastoma Cells

To gain additional insight into the role of endogenous HIF-1α and HIF-2α in VEGF induction by hypoxia, we evaluated the effects of their targeted knockdown by siRNA. siRNA specificity and efficiency, as well as optimal concentrations and kinetics, were determined by Western blot (data not shown). HIF-1α and HIF-2α siRNA selectively counteracted hypoxic induction of the targeted proteins (Fig. 3A), which were decreased by 80% to 85% (*lanes 4* and *6*) and 87% to 93% (*lanes 5* and *6*), respectively, compared with untransfected hypoxic samples (*lane 2*). Control siRNA did not modify expression of either protein (*lane 3*).

HIF-1α/2α silencing significantly reduced VEGF promoter transactivation by hypoxia (Fig. 3B). We observed ≈70% inhibition of hypoxia-induced VEGF-p7-driven luciferase activity after knockdown of either subunit and almost 80% decrease of gene transcription directed by VEGF-p11wt. Concomitant HIF-1α/2α knockdown by the combination of HIF-1α and HIF-2α siRNA resulted in ≈90% suppression of hypoxia-induced VEGF promoter activity. Control siRNA did not modify luciferase activity.

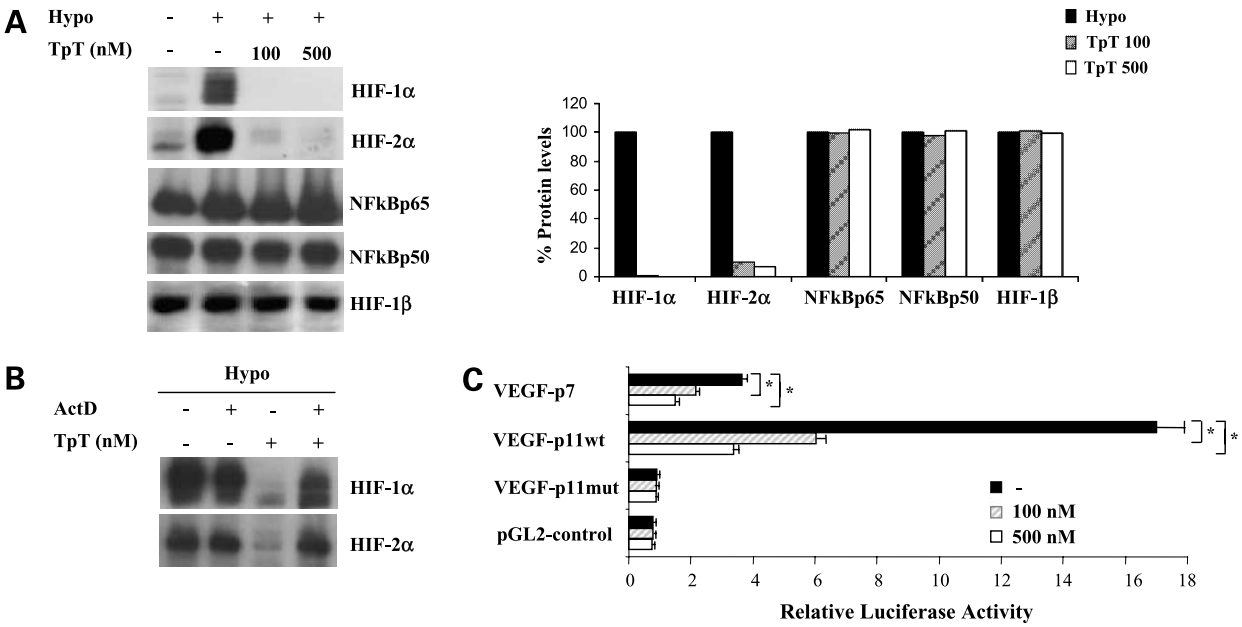

Figure 4. Inhibition by topotecan of hypoxia-induced HIF-1α and HIF-2α expression and HIF-dependent VEGF transcription. **A** and **B,** Western blot analysis of HIF and NF-κB expression. Total extracts from LAN-5 cells were analyzed for HIF-1α/2α, HIF-1β, and NF-κBp65/p50 protein levels after exposure to hypoxia (+) ± 100 or 500 nmol/L topotecan for 6 h, in the presence (**B**) or absence (**A**) of actinomycin D (5 μg/mL), as detailed in Fig. 2B legend. Representative autoradiographs. Data in **A** are also presented as percentage of protein remaining after topotecan treatment relative to untreated hypoxic cells (equal to 100%). **C,** inhibition of VEGF promoter transactivation. LAN-5 cell line was transiently transfected with the indicated luciferase reporter constructs and exposed to hypoxia ± 100 or 500 nmol/L topotecan for 18 h. Cell extracts were then assayed for luciferase activity. Relative luciferase activity of hypoxic versus normoxic cells (considered as equal to 1) is presented as in Fig. 2A. Mean ± SE of five independent experiments. *, $P < 0.01$.

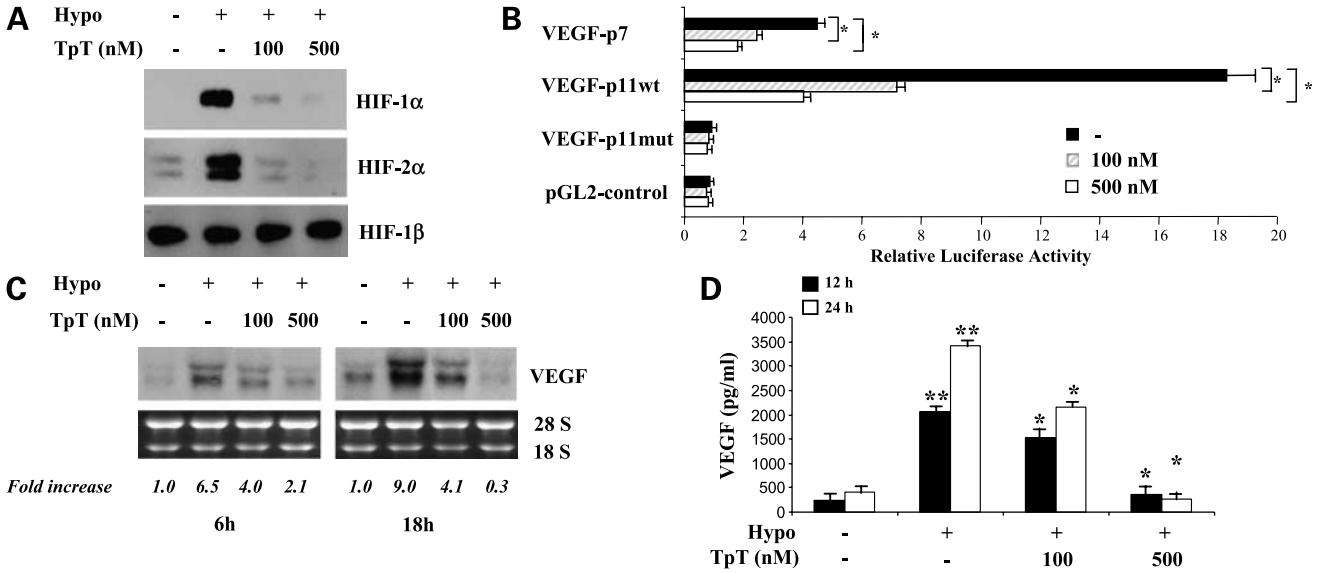

Figure 5. HIF-1α/2α suppression by topotecan mediates inhibition of hypoxia-induced VEGF expression in the GI-LI-N cell line. GI-LI-N cells were treated with 100 or 500 nmol/L topotecan under normoxic or hypoxic conditions. **A,** HIF-1α, HIF-2α, and HIF-1β protein levels were assessed by Western blot as in Fig. 4A. Representative blot of three experiments. **B,** luciferase activity was assayed in cells transfected with the indicated luciferase constructs as detailed in Fig. 4C legend. Mean ± SE of four independent experiments. *, $P < 0.01$. **C,** VEGF mRNA expression was analyzed by Northern blot. Representative of four experiments. VEGF fold increase, determined by densitometric analysis of the band intensities and normalized to the corresponding levels of 18S rRNA, is indicated relative to normoxic values (defined as equal to 1). **D,** conditioned medium was harvested and assayed for the content of secreted VEGF by ELISA. Samples were run in triplicate for each condition. Individual histograms represent mean ± SE (pg/1 × 10^6 cells/mL) of four independent experiments. **, $P < 0.01$ versus respective normoxic levels; *, $P < 0.05$ versus respective hypoxic levels.

These data complement the results obtained by rHIF-α cotransfection (Fig. 2C), suggesting that VEGF transcription activation by hypoxia was dependent on both HIFα subunits and that the stronger rHIF-2α versus rHIF-1α activity observed in cotransfection studies was probably accounted for by higher transgenic HIF-2α expression rather than to preferential promoter recognition or higher transactivation activity, as reported in other studies (37).

VEGF transcription inhibition by HIFα knockdown was paralleled by a significant decrease of hypoxia-induced VEGF mRNA expression as assessed by quantitative real-time PCR (Fig. 3C). VEGF mRNA up-regulation was strongly triggered by hypoxia (~6-fold), confirming Northern blot data, and this induction was reduced by ~45% and ~32% in HIF-1α and HIF-2α siRNA-transfected cells, respectively, but not affected by control siRNA transfection. Concomitant HIF-1α/2α silencing had an additive, although not complete, inhibitory effect on VEGF mRNA (up to 65% reduction).VEGF protein concentrations in conditioned medium from hypoxic cells were decreased to a similar extent following HIF-1α and/or HIF-2α silencing (Fig. 3D). These results indicate that both HIF-1α and HIF-2α contribute to hypoxic induction of VEGF in neuroblastoma.

Topotecan Inhibits HIF-1α and HIF-2α Accumulation and Transcriptional Activity in Hypoxic Neuroblastoma Cells

Recent evidence indicates that camptothecin analogues inhibit HIF-1α induction by hypoxia in glioma (22). However, the effects on HIF-2α expression have not been investigated. We evaluated HIF-1α and HIF-2α protein levels in hypoxic LAN-5 cells treated with the indicated concentrations of topotecan (Fig. 4A). Topotecan (100 nmol/L) completely counteracted hypoxia stimulatory effects not only on HIF-1α but also on HIF-2α subunit within 6 h, decreasing protein expression to levels lower than those of normoxic cells. The expression of other hypoxia-responsive factors involved in the regulation of VEGF transcription, namely NF-κBp65/p50 (6, 38), was then evaluated to assess the specificity of topotecan action on HIFα subunits. HIF-1β protein levels were also analyzed as specificity control (Fig. 4A). All tested proteins were constitutively expressed in LAN-5 cells under normoxia, and 6 h exposure to hypoxia increased NF-κBp65 levels by ≈2-fold. However, treatment with topotecan did not affect NF-κBp65 up-regulation or expression of NF-κBp50 and HIF-1β, suggesting targeted inhibition of HIF-1α/2α proteins by the drug.

Recent observations in glioma cells led to the hypothesis that RNA transcription is essential for topotecan action (39). To provide some insight into the mechanism by which topotecan attenuate HIFα accumulation in hypoxic neuroblastoma, we investigated the role of transcription-mediated events for topotecan-inhibitory activity by treating LAN-5 cells with the RNA transcription inhibitor, actinomycin D, before addition of 500 nmol/L topotecan for 6 h under hypoxic conditions (Fig. 4B). The blockade of RNA transcription almost completely reversed topotecan ability to inhibit HIF-1α and HIF-2α, without affecting hypoxic

accumulation of either protein, implicating the requirement of ongoing RNA transcription for the drug-inhibitory effects on both subunits in neuroblastoma.

Experiments were then carried out to determine whether HIF-1α and HIF-2α inhibition by topotecan was associated with suppression of hypoxia-induced VEGF transcription (Fig. 4C). VEGF promoter transactivation (*solid columns*) was significantly inhibited by topotecan, with ~60% and 80% decrease of VEGF-p7-driven luciferase expression exerted by 100 nmol/L (*hatched columns*) and 500 nmol/L (*open columns*), respectively, and 70% and 85% inhibition achieved in VEGF-p11wt-transfected cells. VEGF-p11mut construct and pGL2-control vector were not responsive to hypoxia, and their basal activity was not affected by topotecan. We conclude that topotecan represses VEGF gene transcription in hypoxic neuroblastoma cells by targeting HIF-1α and HIF-2α.

To confirm that topotecan-inhibitory activity was not unique to LAN-5 cells but could be extended to other neuroblastoma cell lines, we tested the drug effects on GI-LI-N cells. Similarly to what shown in LAN-5, HIF-1α and HIF-2α induction by hypoxia was almost completely abrogated within 6 h treatment with 100 nmol/L topotecan (Fig. 5A), whereas HIF-1β was not affected. HIFα inhibition was associated with suppression of VEGF promoter transactivation (Fig. 5B). Culture under hypoxic conditions (*solid columns*) resulted in ≈4- and 18-fold increase in luciferase activity directed by VEGF sequences, respectively, in the context of the native (VEGF-p7) or the heterologous (VEGF-p11wt) promoters, and HIF-1-binding site mutation (VEGF-p11mut) abrogated reporter gene inducibility by hypoxia. Topotecan at 100 nmol/L (*hatched columns*) and 500 nmol/L (*open columns*) decreased hypoxia effects by 59% and 78%, respectively, in VEGF-p7-transfected cells and by 71 and 85% in VEGF-p11wt-transfected cells, whereas luciferase activity driven by the pGL2-control vector was unaffected by hypoxia or topotecan. Transcription inhibition was paralleled by reduction of VEGF mRNA expression (Fig. 5C) and protein secretion (Fig. 5D). Transcript up-regulation by hypoxia was decreased by 45% and 80% on addition of 100 and 500 nmol/L topotecan for 6 h, and complete mRNA inhibition was achieved at a dose of 500 nmol/L after 18 h. Accordingly, secreted VEGF in conditioned medium from hypoxic cells was significantly reduced by topotecan, and almost complete inhibition achieved by 500 nmol/L already after 12 h. These data confirm that HIF-1α and HIF-2α suppression by topotecan mediates the drug-inhibitory activity on VEGF production by hypoxic neuroblastoma cells.

Topotecan Counteracts Desferrioxamine-Induced HIF-1α Expression Resulting in Decreased VEGF Production

HIF-1α and HIF-2α are inducible under normal pO_2 by several hypoxia-mimetic stimuli (6, 17, 18, 35, 40). We reported that the iron chelator, desferrioxamine, could

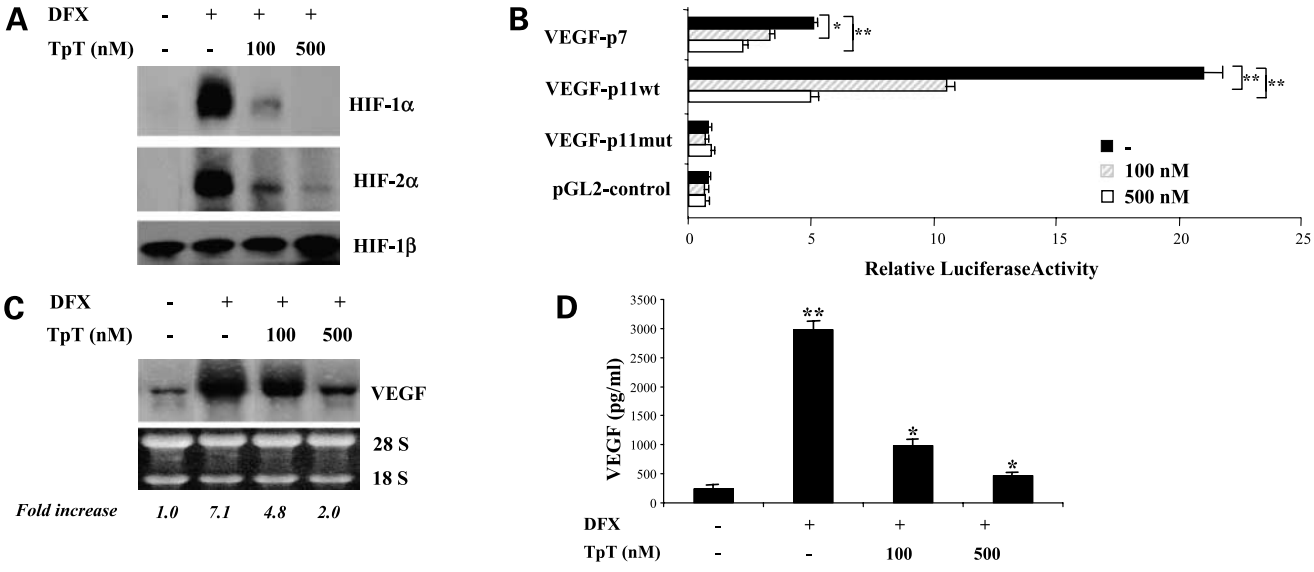

Figure 6. Topotecan-dependent inhibition of desferrioxamine-induced HIF-1α/2α and VEGF production by neuroblastoma cells. **A,** inhibition of HIF-1α and HIF-2α accumulation. LAN-5 cells were treated with 100 μmol/L desferrioxamine (+) for 6 h in the presence or absence of 100 and 500 nmol/L topotecan, and HIF-1α and HIF-2α immunodetection was done by Western blot as described in Fig. 2B legend. Representative of three independent experiments. **B,** inhibition of HIF-driven VEGF promoter transactivation. Luciferase activity was assayed in LAN-5 cells transfected with the indicated luciferase reporter constructs after 18 h treatment with 100 μmol/L desferrioxamine ± 100 or 500 nmol/L topotecan. Data are presented as relative luciferase activity of desferrioxamine-treated versus untreated cells (considered as equal to 1). Mean ± SE of four independent experiments. *, $P < 0.05$; **, $P < 0.01$. **C,** VEGF mRNA inhibition. Total RNA was isolated from LAN-5 cells treated for 18 h with 100 μmol/L desferrioxamine in the presence or absence of the indicated doses of topotecan and analyzed for VEGF mRNA expression by Northern blot as described in Fig. 1A. Representative of three experiments. VEGF fold increase is indicated relative to untreated cells (defined as equal to 1). **D,** supernatants were assayed for VEGF content by ELISA after 24 h treatment with 100 μmol/L desferrioxamine in the presence or absence of 100 or 500 nmol/L topotecan. Mean ± SE (pg per 1×10^6 cells/mL) of three independent experiments. **, $P < 0.01$ versus untreated cells; *, $P < 0.01$ versus desferrioxamine alone.

trigger VEGF gene expression in advanced-stage neuroblastoma cells (15). We evaluated whether topotecan inhibited desferrioxamine stimulatory effects and the mechanisms involved. LAN-5 cell treatment with 100 μmol/L desferrioxamine for 6 h strongly up-regulated both HIFα subunits (Fig. 6A), resulting in VEGF promoter transactivation (Fig. 6B, *solid columns*), with luciferase activity increased to levels comparable with those induced by hypoxia (5- and 21-fold above those of untreated cells on VEGF-p7 and VEGF-p11wt transfection, respectively). VEGF transcription required the integrity of the HIF-binding site, because it was abolished by its targeted mutation (VEGF-p11mut; Fig. 6B) and was paralleled by significant VEGF mRNA up-regulation (Fig. 6C) and increased protein secretion (≈12-fold) (Fig. 6D). These data confirm the ability of desferrioxamine to mimic hypoxia stimulatory effects on VEGF expression under normal pO_2 by inducing HIF-1α and HIF-2α expression and activity. Consistent with the results obtained for hypoxia, addition of topotecan strongly counteracted desferrioxamine-dependent HIF-1α/2α induction (Fig. 6A) and HIF-dependent VEGF promoter transactivation (Fig. 6B), decreasing luciferase levels by 45% and 73% in cells transfected with VEGF-p7 at 100 nmol/L (*hatched columns*) and 500 nmol/L (*open columns*), respectively, and by 55% and 80% in VEGF-p11wt-transfected cells. Accordingly, a dose-dependent inhibition of VEGF mRNA (Fig. 6C) and protein (Fig. 6D) was induced by topotecan, with almost complete abrogation of desferrioxamine stimulatory effects achieved with 500 nmol/L. Under these experimental conditions, topotecan did not affect the expression of rRNA 28S and 18S. Similar results were observed in three independent experiments, suggesting that topotecan inhibits HIF-1α/2α induction by hypoxia-mimetic agents under normoxia in neuroblastoma, leading to decreased VEGF production.

Discussion

High VEGF expression in neuroblastoma tumors is a marker of poor prognosis (7, 28), and VEGF inhibition has potential therapeutic implications for neuroblastoma treatment (8, 16). In this study, we identify topotecan as a strong inhibitor of VEGF induction by hypoxia in two neuroblastoma cell lines derived from advanced-stage tumors and of their angiogenic activity *in vivo*. Moreover, we provide the first evidence that topotecan targets the expression and transcriptional activity of both HIF-1α and HIF-2α subunits as a mechanism to counteract hypoxic stimulation of VEGF.

VEGF mRNA inhibition by topotecan occurred rapidly, within 6 h treatment, and was dose dependent, with the range of effective concentrations (100-500 nmol/L) not cytotoxic and comparable with those active on glioma cells (22), indicating that topotecan can suppress responses to hypoxia in different types of tumors. The functional relevance of these findings is underlined by the demonstration that VEGF mRNA inhibition was associated with suppression of protein secretion and with significant reduction of the *in vivo* angiogenic potential of hypoxic neuroblastoma cells. The fact that the effective antiangiogenic doses of topotecan correspond to clinically achievable concentrations (26) suggests potential therapeutic implications of these findings.

Transcription regulation plays a major role in the hypoxic control of VEGF expression in different cell types (6, 22, 34, 41, 42), and we extend this observation to neuroblastoma cells. Promoter-driven reporter studies showed that 1-kb fragment of the VEGF 5′-flanking sequences was able to mediate transcriptional responses to hypoxia and that a 47-bp region located between −985 and −939 from the transcription start site was sufficient for transactivation of a heterologous promoter. Mutational analysis of this hypoxia-inducible enhancer revealed the requirement of the HRE at position −975 for transcription, suggesting that the HIF pathway is involved in VEGF hypoxic up-regulation in neuroblastoma.

The hypoxia-sensitive HIF-1α and HIF-2α subunits are frequently coexpressed in human tumors and implicated in the regulation of genes involved in proliferation, angiogenesis, and glucose metabolism (9, 17–21, 38). However, their respective roles in the control of hypoxia target genes have not been fully clarified. Functional differences between the two α-subunits and specificity in the pattern of gene expression were suggested by knockout and RNA interference experiments (19, 33, 37, 42), whereas transgene approaches indicated partial functional redundancy, lack of target gene specificity, and cell type–specific and/or temporospatial-dependent regulation of the same subset of hypoxia-driven genes (28, 33, 37, 41, 42). In this study, we found that hypoxic neuroblastoma cells expressed both α-subunits, but with a different kinetics of induction, being HIF-1α rapidly and transiently stabilized and HIF-2α more gradually and continuously accumulated, suggesting a primary role for HIF-1α in mediating acute gene activation by hypoxia and the involvement of HIF-2α during prolonged hypoxia in neuroblastoma. Furthermore, we show that the two subunits similarly contribute to VEGF transcriptional activation in hypoxic neuroblastoma cells, because (*a*) exogenous overexpression of either protein resulted in VEGF promoter transactivation under normoxia, both in the context of the native and the heterologous promoters, which was abrogated by HIF-1-binding site mutation and further increased under hypoxia, and (*b*) targeted knockdown of either subunit by RNA interference counteracted hypoxia ability to activate VEGF transcription. These results are consistent with recent observations in other neuroblastoma cell lines (28) and extend the findings obtained with human embryonic kidney cells (42).

The simplest explanation for the dramatic inhibitory effects of silencing HIF-1α/2α alone on VEGF transcription is that the total amounts of HIFα proteins have to reach a certain threshold to trigger VEGF promoter transactivation. Silencing the expression of either subunit decreases the levels of protein available below the critical threshold, resulting in transcription inhibition. Alternatively, it is possible that HIF-1α and HIF-2α act in concert to promote

VEGF transcription and that silencing either of them prevents response to hypoxia. The requirement of both subunits for hypoxia responsiveness has been suggested previously (42). The finding that concomitant HIF-1α/2α knockdown almost completely abrogated hypoxia-induced VEGF promoter activity, whereas inhibition of VEGF mRNA and protein expression was more modest raises the possibility that the HIF/HRE system is primarily, but not entirely, implicated in hypoxic inducibility of VEGF in neuroblastoma cells and that signaling pathways involving other hypoxia-responsive factors (20, 38) binding to promoter regions not comprised in the luciferase constructs used in this work (43) may contribute to this effect. Future studies to identify these HIF/HRE-independent regulatory mechanisms are warranted.

The degree of local oxygenation within solid tumors is quite heterogeneous and rapidly fluctuating, ranging from 5% O_2 in well-vascularized regions to 1% O_2 near necrotic areas (10, 44). Signals other than hypoxia, such as genetic alterations, transition metals, chelating agents, hormones, and growth factors, share with hypoxia the property of inducing HIF-1α/2α overexpression and HIF-dependent gene transcription via HRE under normal pO_2 in various cell types (6, 17, 18, 35, 40, 41). The identification of pharmacologic inhibitors of the HIF pathway active under both hypoxic and normoxic pO_2 is a major goal of current therapeutic strategies for cancer treatment (17, 22, 23). Topotecan has been recently identified as an efficient inhibitor of HIF-1α induction by hypoxia in glioma cells by acting on mRNA translation (22, 39). Subsequent studies in neuroblastoma showed that steady-state and growth factor–stimulated HIF-1α expression under normoxic conditions was also targeted by topotecan (40). Our findings extend these observations by providing the first evidence that topotecan can inhibit not only HIF-1α but also HIF-2α expression and activity induced by hypoxia as well as by the hypoxia-mimetic agent, desferrioxamine (35), in neuroblastoma cells, resulting in the suppression of HIF-driven VEGF transcription and to decreased VEGF production. Taken together, these findings clearly indicate that topotecan is an inhibitor of both HIF-1α- and HIF-2α-mediated responses in neuroblastoma at pathologic and physiologic O_2 concentrations. The novel finding of HIF-2α targeting by topotecan is of potential therapeutic relevance, given recent evidence of its continuous accumulation and transcriptional activity *in vitro* in neuroblastoma cultures under hypoxic and normoxic conditions and *in vivo* in both hypoxic and well-vascularized areas of neuroblastoma specimens and of the implication of this subunit, rather than HIF-1α, as the major determinant of neuroblastoma tumor angiogenesis, aggressiveness, and unfavorable patient outcome (21, 28).

Interestingly, topotecan-inhibitory action appeared to have a certain degree of specificity on HIFα subunits, because the expression of HIF-1β, as well as that of NF-κBp65 and NF-κBp50, two hypoxia-responsive transcription factors involved in the regulation of VEGF transcription (6, 38, 45–47) through a NF-κB-like response element at position −650/−635 of the VEGF promoter (48), was not affected by treatment with the same doses of topotecan active on HIFα accumulation in hypoxic neuroblastoma cells. Additional studies are clearly required to assess the possibility that topotecan may target other transcription factors mediating hypoxia-regulated VEGF expression (6, 38). Our data also implicate the requirement of ongoing RNA transcription for topotecan-inhibitory activity on HIF-1α/2α subunits in neuroblastoma, confirming previous observations on HIF-1α in glioma cells (39) and suggesting the existence of a common mechanism of inhibition of both subunit accumulation by the drug, which can be exerted in different tumor cell types. Studies are ongoing to assess whether topoisomerase I poisoning by topotecan may induce transcriptional activation of a mediator responsible for HIFα protein inhibition or whether the presence of topoisomerase I-DNA cleavage complexes on the transcribed strand of active genes may arrest the RNA transcription machinery generating a signal that ultimately blocks HIFα subunit accumulation.

In conclusion, we show that topotecan exerts a potent antiangiogenic activity on neuroblastoma cells through suppression of both acute (HIF-1α-dependent) and prolonged (HIF-2α-dependent) VEGF induction by hypoxia. Preclinical evidence by Kim et al. showed that ''low-dose pulse'' topotecan significantly reduced angiogenesis and tumor growth *in vivo* in a neuroblastoma xenograft model (49), and a later study by Rapisarda et al. showed similar effects in a glioma xenograft model on daily administration of low doses of topotecan, which were associated with concomitant inhibition of HIF-1α and HIF-1 target gene expression *in vivo* (27). These findings provide a rationale for using topotecan in protracted, low-dose clinical regimens aimed at inhibiting HIFα function in neuroblastoma, which have the potential to be effective in the control of tumor angiogenesis and progression.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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