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Advances in Brief

Schedule-dependent Inhibition of Hypoxia-inducible Factor-1α Protein Accumulation, Angiogenesis, and Tumor Growth by Topotecan in U251-HRE Glioblastoma Xenografts

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Abstract

We have previously shown that topotecan, a topoisomerase I poison, inhibits hypoxia-inducible factor (HIF)-1α protein accumulation by a DNA damage-independent mechanism. Here, we report that daily administration of topotecan inhibits HIF-1α protein expression in U251-HRE glioblastoma xenografts. Concomitant with HIF-1α inhibition, topotecan caused a significant tumor growth inhibition associated with a marked decrease of angiogenesis and expression of HIF-1 target genes in tumor tissue. These results provide a compelling rationale for testing topotecan in clinical trials to target HIF-1 in cancer patients.

Introduction

Over the last several years, hypoxia-inducible factor 1 (HIF-1) has emerged as an attractive target for cancer therapy (1, 2). HIF-1 plays a pivotal role in the regulation of key survival pathways in cancer cells by activating the transcription of genes involved in angiogenesis, tumor metabolism, migration, and invasion (1). HIF-1 is a basic helix-loop-helix PAS (Per-Arnt-Sim) transcription factor composed of an α subunit, whose levels are tightly regulated by changes in oxygen concentration, and a β subunit, which is constitutively expressed (3). Overexpression of HIF-1α protein has been reported in several human cancers (4), where it has been associated with tumor progression, treatment failure, and poor survival (5–7).

Topotecan, a camptothecin analogue, is an S-phase–specific agent that causes cytotoxicity by a mechanism dependent on DNA replication-mediated DNA damage (8). Interestingly, protracted administration of topotecan appears to be more efficacious both in animal models and human cancers (9–12), although the mechanism underlying this phenomenon remains poorly understood. In addition, topotecan may have an antiangiogenic effect by inhibiting endothelial cell proliferation (13, 14), but the contribution of this action to the therapeutic activity of topotecan is unknown.

Topotecan inhibits HIF-1α protein accumulation in human cancer cell lines (15, 16) independently of DNA replication-mediated DNA damage, raising the possibility of a mechanism of action distinct from the one responsible for the cytotoxic effects. In this report, we show that topotecan administered on a daily but not an intermittent schedule caused a sustained inhibition of tumor growth in U251-HRE xenografts. The antitumor activity of topotecan was associated with a marked decrease of HIF-1α protein levels, angiogenesis, and the expression of HIF-1 target genes in tumor tissue, relative to vehicle controls.

These results provide the first evidence that topotecan inhibits HIF-1α protein in human xenografts on a schedule that is associated with inhibition of angiogenesis and tumor growth.

Materials and Methods

Cell Lines and Reagents. U251 human glioma cells were maintained as described previously (16). U251-HRE and U251-mutHRE express luciferase under control of three copies of wild-type or mutant HRE, respectively (16). Experiments under hypoxia (1% O_2) were performed in the hypoxic workstation INVIVO$_2$ 400 (Biotrace International, Cincinnati, OH). Topotecan was obtained from the Drug Synthesis and Chemistry Branch, Developmental Therapeutics Program, National Cancer Institute.

Animal Studies and Imaging. All studies were conducted in female athymic nude (NCr/nu) mice obtained from the Animal Production Area (National Cancer Institute–Frederick) in an Assessment and Accreditation of Laboratory Animal Care-accredited facility with an approved animal protocol. Tumors were generated with the U251-HRE and the U251-mutHRE cell lines by injecting 1×10^7 tumor cells s.c. into the flank. Tumor size was monitored by collecting length and width measurements and calculating the tumor weight (mg) as $\{[\text{tumor length} \times (\text{tumor width})^2]/2\}$. For luminescence imaging, mice received 150 mg of firefly luciferase (Biosynth AG, Staad, Switzerland) per kg body weight given i.p. After anesthesia with isoflurane gas (Abbott Laboratories, North Chicago, IL), the mice were placed into a Xenogen IVIS imaging station (Xenogen Corp., Alameda, CA) and imaged using Living Image Software (Xenogen Corp.).

Topotecan, solubilized in sterile water, was dosed i.p., and sterile water was the vehicle control.

Immunoblotting Analysis. Western blotting analysis from whole cell lysates was performed as described previously (15). Monoclonal anti-HIF-1α and anti-β-actin antibodies were purchased from BD Transduction Laboratories (Lexington, KY) and from Chemicon International, Inc. (Temecula, CA), respectively.

Real-Time PCR. Total RNA from tumors was isolated using the RNA Mini kit (Qiagen, Inc., Valencia, CA) accordingly to the manufacturer's procedure. Reverse transcription-PCR and real-time PCR to measure human vascular endothelial growth factor, human phosphoglycerate kinase 1, human ornitine decarboxylase, and mouse vascular endothelial growth factor mRNA expression were performed as described previously (16). 18S rRNA, as internal control, was assessed using premixed reagents from Applied Biosystems. The sequence of primers and probes used is available upon request.

Immunohystochemistry. Tumors were fixed in 4% paraformaldehyde and then processed and embedded in paraffin. HIF-1α antigen retrieval was performed in target retrieval solution (DakoCytomation California, Inc., Carpinteria, CA). Monoclonal anti-HIF-1α antibody was used at a dilution of 1:25 (BD Transduction Laboratories). Monoclonal antibody anti PECAM-1 was

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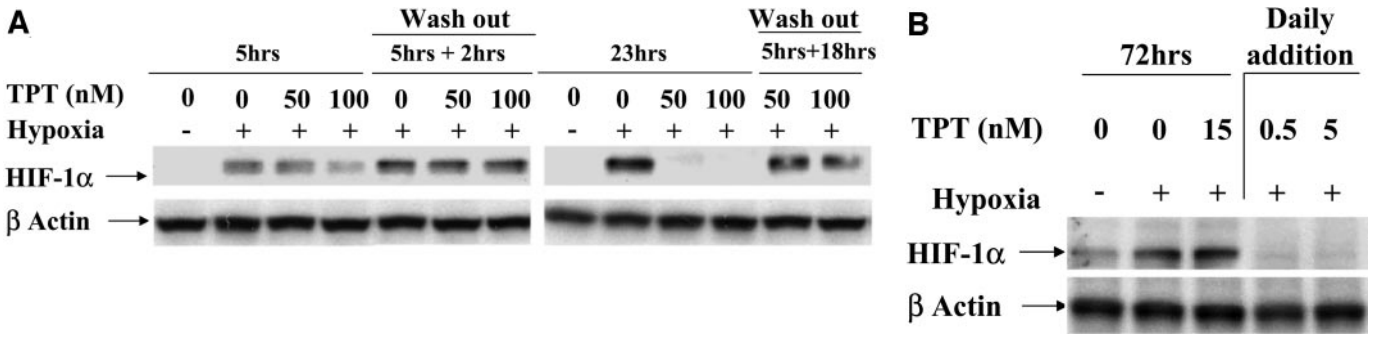

Fig. 1. Inhibition of HIF-1α protein accumulation by topotecan (TPT) is reversible and schedule dependent. *A.* U251 cells were cultured under normoxia or hypoxia in the absence or presence of TPT for 5 hours. After washing with PBS under hypoxic conditions, cells were incubated, with or without TPT, for an additional 2 or 18 hours. Results are representative of two independent experiments. *B.* U251 cells were cultured for 72 hours under normoxic or hypoxic conditions in the presence of the indicated concentrations of TPT, which was added either once on day 1 or daily (every 24 hours) for a total of three times. Results are representative of two independent experiments.

used at a dilution of 1:100 (Santa Cruz Biotechnology, Inc., Santa Cruz, CA). Detection was performed by standard avidin-biotin complex methods.

Results

***In vitro* Inhibition of Hypoxia-Inducible Factor–1α Protein Accumulation by topotecan Is Reversible and Schedule Dependent.** We first determined whether the inhibition of HIF-1α protein accumulation by topotecan was reversible. Topotecan (50 to 100 nmol/L) partially or completely inhibited hypoxic induction of HIF-1α protein accumulation at 5 and 23 hours, respectively (Fig. 1*A*). Interestingly, HIF-1α inhibition was completely reversible as early as 2 hours and, even more dramatically, 18 hours after removal of topotecan from the media in cells maintained under hypoxic conditions. These results suggest that continuous presence of topotecan is required for the inhibition of HIF-1α protein accumulation.

Next, we tested whether low concentrations of topotecan (0.5 to 5 nmol/L) administered on a daily basis were sufficient to inhibit HIF-1α protein accumulation. As shown in Fig. 1*B*, a single exposure of topotecan up to 15 nmol/L did not inhibit hypoxia-induced HIF-1α protein accumulation after 72 hours of incubation. In contrast, daily addition of topotecan (0.5 or 5 nmol/L) to hypoxic-treated cells completely abrogated HIF-1α protein expression at 72 hours, indicat-

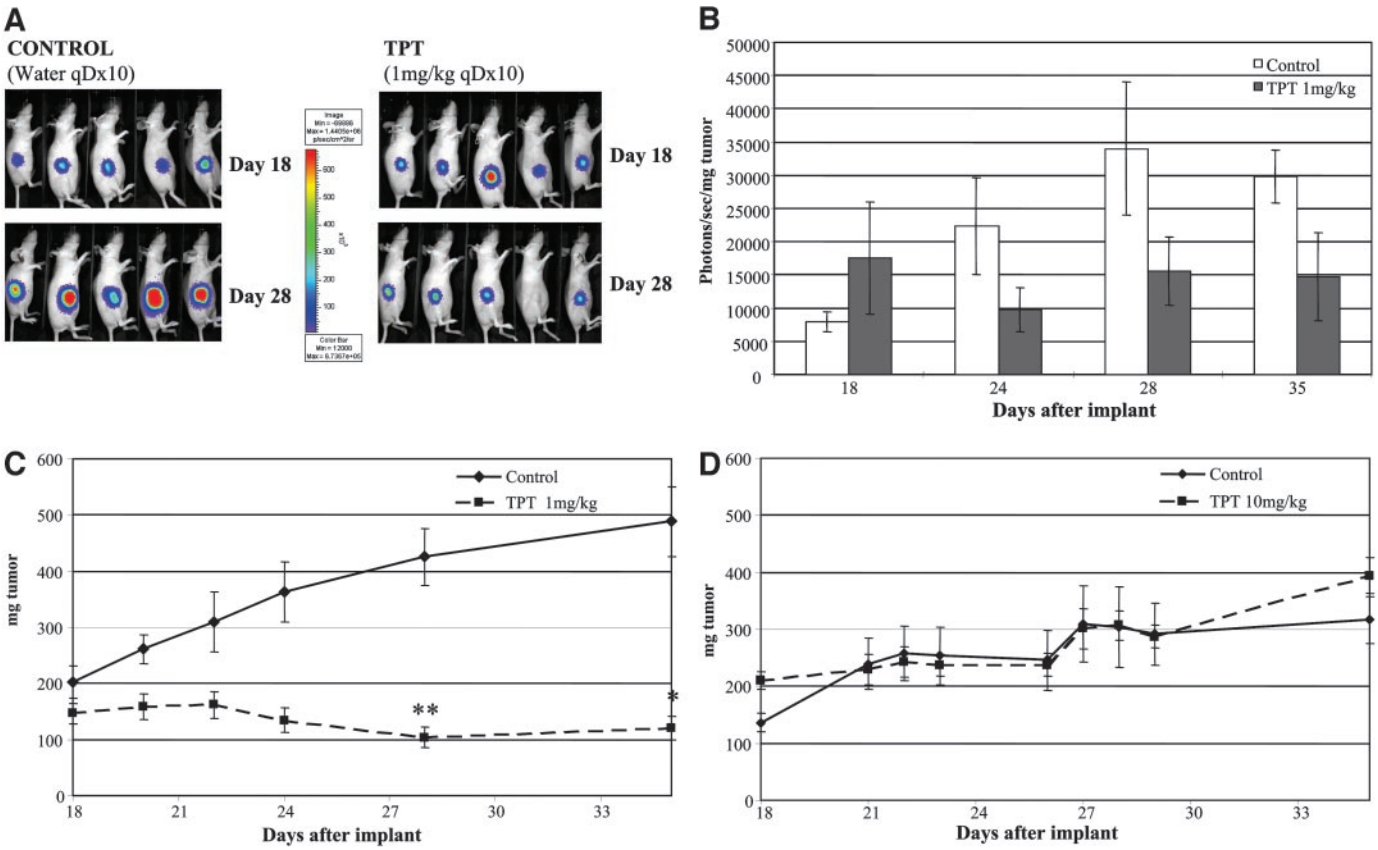

Fig. 2. Daily administration of topotecan (TPT) inhibits HRE-dependent luciferase expression and tumor growth in U251-HRE xenografts. *A.* U251-HRE cells were implanted in nude mice (five per group) and allowed to grow up to day 18 when treatment was started as indicated. Luminescence was measured as described in Materials and Methods. On day 28 (end of treatment), there was a statistically significant reduction of luminescence (photons per second) in mice treated with TPT, relative to controls (Mann-Whitney test, $P < 0.008$). *B.* Luminescence was measured on the indicated days from mice treated with water or TPT 1 mg/kg qd×10, and values were normalized for tumor mass. The difference on day 28 was not statistically significant. *C* and *D.* Tumor weights were recorded daily in mice treated with vehicle or TPT (1 mg/kg qd×10; *C*) or TPT (10 mg/kg q4d×3; *D*). Statistical analysis showed a significant inhibition of tumor growth in mice treated with daily TPT (Mann-Whitney test, $P < 0.008$), relative to untreated controls.

ing that daily administration of low doses of topotecan might be necessary and sufficient to achieve sustained inhibition of HIF-1α protein accumulation.

Daily Administration of topotecan Inhibits HRE-dependent Luciferase Expression and Tumor Growth in U251-HRE Xenografts. The U251-HRE, but not U251-mutHRE (data not shown), cell line implanted s.c. in mice emitted detectable luminescence that increased with increasing tumor size, demonstrating that luciferase expression in U251-HRE xenografts is dependent on the presence of a functional HRE sequence.

We then designed experiments to test whether topotecan inhibited HIF-1–dependent luciferase expression and tumor growth in U251-HRE xenografts. When tumors reached ~175 mg in size, the mice were randomized into treatment groups ($n = 5$ or 10 per group). Topotecan was administered according to two different regimens: daily [1 mg/kg once daily for 10 doses (qd×10)] or intermittent (10 mg/kg q4d×3). As shown in Fig. 2*A*, the vehicle (qd×10)-treated mice had progressive increases in luminescence, whereas the topotecan (qd×10)-treated mice had diminished luminescence from the start of treatment (day 18) to the end (day 28). Because the tumor mass was reduced by topotecan treatment, the luminescence values were normalized for tumor mass. As shown in Fig. 2*B*, the normalized luminescence in the control group increased from day 18 to day 28. In contrast, the topotecan-treated mice had a modest decrease in the normalized luminescence during the same time frame. Intermittent administration of topotecan caused a transient inhibition of luminescence 24 hours after each dose compared with untreated controls (data not shown).

Notably, daily but not intermittent administration of topotecan had a marked effect on tumor growth. As shown in Fig. 2*C*, the tumor weights of the mice treated with 1 mg topotecan/kg on the qd×10 schedule were significantly reduced compared with the control mice ($P < 0.008$). In contrast, 10 mg topotecan/kg given q4d×3 did not impact the tumor weights compared with the control mice (Fig. 2*D*). These results demonstrate that topotecan manifested a schedule-dependent antitumor activity in U251-HRE xenografts.

Daily Administration of topotecan Decreases Microvessel Density, Hypoxia-Inducible Factor-1α Expression, and Hypoxia-Inducible Factor-1 Target Genes in U251-HRE Xenografts. Tumor sections from animals treated with daily topotecan showed a decrease in tumor cellularity and an increase in extracellular matrix deposition, relative to controls (Fig. 3, *A* and *B*). CD31+ staining in tumor sections from control animals showed the presence of a large number of vessels, which were dramatically decreased in number and size in mice treated with daily topotecan (Fig. 3, *C* and *D*; $P < 0.009$). Concordant with the pattern of blood vessels observed, tumors from mice treated with daily topotecan showed a dramatic decrease in HIF-1α protein staining (Fig. 3, *E* and *F*), with only sparse and isolated HIF-1α–positive cells remaining, demonstrating that topotecan does decrease HIF-1α protein accumulation *in vivo*.

Consistent with the inhibition of HIF-1α protein, we found that expression of human vascular endothelial growth factor and human

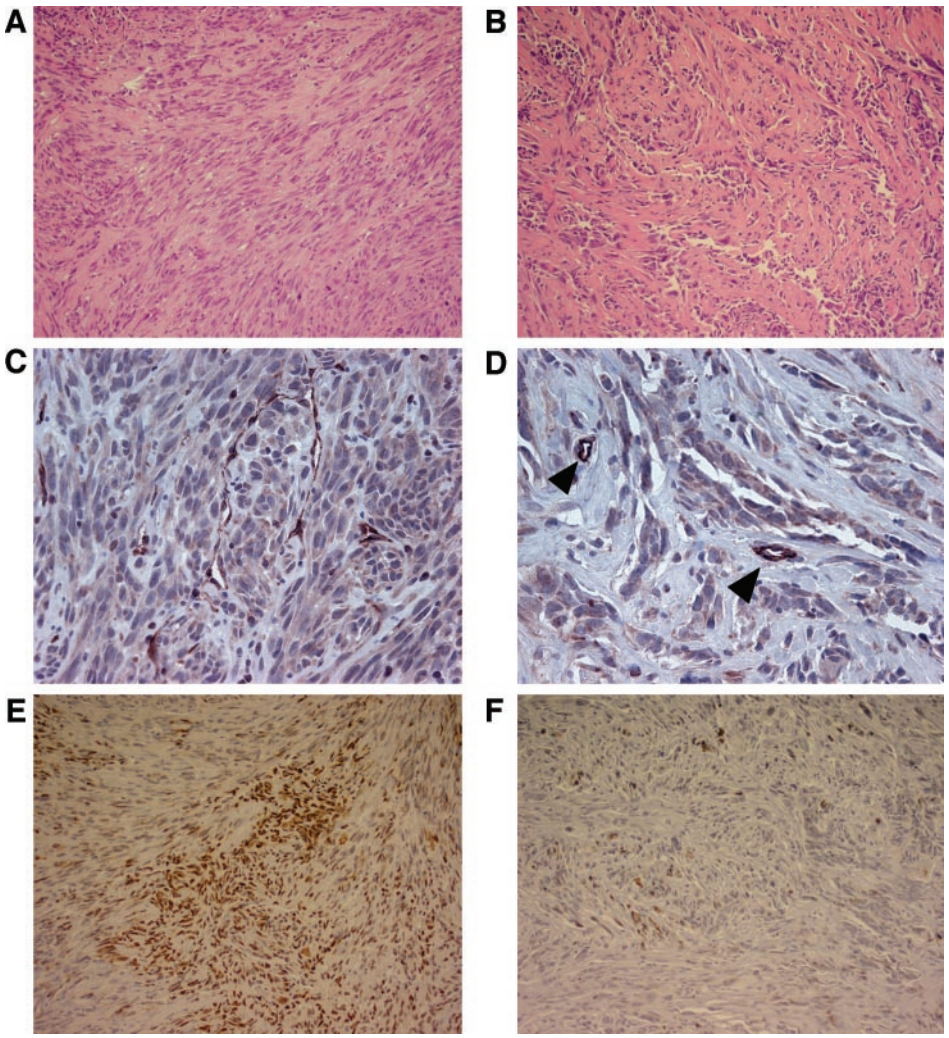

Fig. 3. Topotecan inhibits HIF-1α protein accumulation and microvessel density in U251-HRE xenografts. *A* and *B*, H&E (×200) stain of representative regions of a xenograft of control animal (*A*) showing spindle like growth pattern and (*B*) xenograft treated with 1 mg/kg topotecan with increased extracellular matrix. *C* and *D*, CD31 stain (×250) of representative regions of control (*C*) with well-formed vessels and bifurcations and (*D*) treated with 1 mg/kg topotecan with fewer and smaller vessels. *E* and *F*, HIF-1α stain (×200) of controls (*E*) or mice treated with 1 mg/kg TPT (*F*).

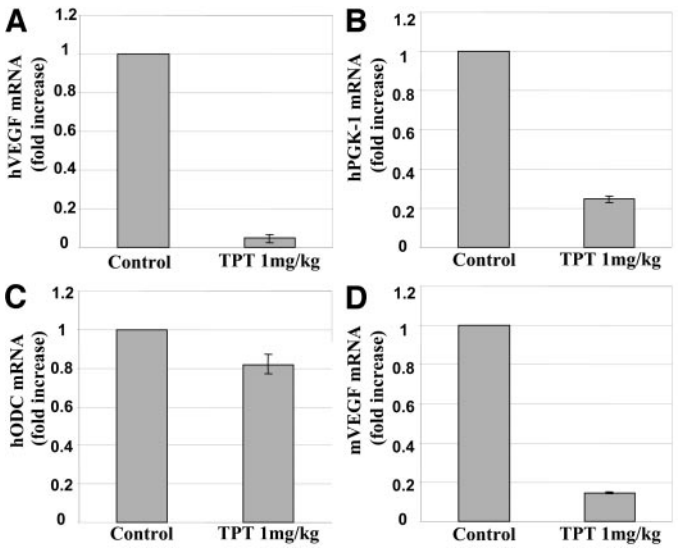

Fig. 4. Topotecan (TPT) inhibits the expression of HIF-1–inducible genes in U251-HRE xenografts. Analysis of mRNA expression for human vascular endothelial growth factor (hVEGF; *A*), human phosphoglycerate kinase 1 (PGK-1; *B*), human ornitine decarboxylase (ODC; *C*), and mouse (m)VEGF (*D*) from tumor lysates of mice treated with vehicle or 1 mg/kg TPT. Results are presented as mean ± SE ($n = 5$).

phosphoglycerate kinase 1 mRNA was decreased by 95 and 90%, respectively, in samples from mice treated with daily topotecan compared with untreated controls (Fig. 4, *A* and *B*). Interestingly, expression of human ornitine decarboxylase mRNA, a hypoxia but not HIF-1–inducible gene, was not significantly affected by treatment with topotecan (Fig. 4*C*). Daily but not intermittent (data not shown) administration of topotecan also inhibited mouse vascular endothelial growth factor mRNA expression, relative to untreated controls (Fig. 4*D*), raising the intriguing possibility that this activity might contribute to the observed therapeutic response.

These findings demonstrate that topotecan inhibits HIF-1α protein accumulation, vessel density, and HIF-1 target genes in U251-HRE xenografts.

Discussion

It has been postulated that the activity observed when topotecan is administered on a chronic schedule is dependent, at least in part, upon the protracted exposure of cancer cells to the drug, which increases the likelihood of targeting S-phase–replicating cells (9–12). Results shown here are consistent with the possibility that daily administration of topotecan causes inhibition of HIF-1α protein, which strongly correlates with inhibition of vascularity and tumor growth. The dramatic difference observed in terms of tumor growth inhibition between the two schedules used in this study is also suggestive of a sustained (daily) *versus* transient (intermittent) inhibition of HIF-1α and microvessel density, which may translate into the presence or absence of clinically meaningful responses. This conclusion is strongly supported by our finding that inhibition of HIF-1α protein is rapidly reversible upon removal of topotecan from cultured cells. Along with the known short half-life of topotecan in humans (8), these results emphasize that chronic administration of topotecan may be required to achieve sustained inhibition of HIF-1.

The causal correlation between HIF-1α inhibition and decrease in microvessel density and tumor growth remains to be further investigated. It is formally possible that inhibition of tumor growth may lead to a consequent decrease of intratumor hypoxia, of which microvessel density and HIF-1α levels might be an epiphenomenon. However, the predominant cytostatic effect observed with the daily regimen, the lack of therapeutic effect of the intermittent schedule (10 mg/kg), and the nice correlation between inhibition of HIF-1α– and HIF-1–inducible genes in tumor tissues argue against the cytotoxic effect being the sole determinant of the therapeutic response. Genetic models in tumor cells that lack HIF-1α or are resistant to topotecan, as well as activity in other tumor xenografts, will further clarify this issue.

The role of HIF-1 inhibitors in cancer therapy remains speculative at this time because selective HIF-1 inhibitors are just emerging. Findings reported here provide a compelling rationale for clinical trials of topotecan aimed at targeting HIF-1 activity in cancer patients.

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