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Augmenting tumor sensitivity to topotecan by transient hypoxia

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Abstract We examined how the effect of topotecan is modulated by transient hypoxia in three different tumor lines, Lewis lung carcinoma (LLC), U87 human glioblastoma and DMS273 human small cell lung cancer. Four groups of tumor bearing mice were treated with saline or a single dose of topotecan, immediately followed by 6-h or 72-h exposure to a hypoxic environment (10% O_2) or normal air. Topotecan + hypoxia resulted in significantly greater suppression of tumor growth than normoxic topotecan or hypoxia alone. Correspondingly, the sensitivity of LLC cells to topotecan in a clonogenic survival assay was significantly higher under hypoxia. This effect of hypoxia was not a general phenomenon, since the tumor growth inhibitory effect of the alkylating agent cisplatin was not changed by hypoxic environment. In a parallel series of in vitro experiments, cell cultures were exposed to hypoxia (0.1% or 0.7% O_2) in a hypoxic chamber or normoxia for 24 h. We found a dose-dependent downregulation of HIF-1α by topotecan (30–270 nM). The hypoxic upregulation of Glucose transporter-1 and VEGF secretion to the culture medium was inhibited by the addition of topotecan, while doses up to 270 nM had no effect on VEGF under normoxia. VEGF protein levels in tumors were also reduced by topotecan. These data show that the effect of topotecan is increased by transient hypoxia, and this may be a direct effect on the ability of cells to survive under hypoxia as well as an antiangiogenic effect, mediated through the HIF-1 inhibitory effect of topotecan.

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Introduction

Topotecan inhibits cell proliferation through inhibition of DNA replication. It has recently been shown to also inhibit the Hypoxia Inducible Factor 1 (HIF-1) transcriptional activation pathway [1] through inhibition of the translation of HIF-1α [2]. Topotecan is a water-soluble derivative of camptothecin that binds to the nuclear enzyme DNA topoisomerase I [3]. Topoisomerase I alters the DNA supercoiling by cleaving a single strand of the DNA duplex. Topoisomerase I is found in transcription and replication complexes, suggesting that it provides the "DNA swivel" required for DNA tracking by RNA and DNA polymerases. It engages in an intimate interaction with its DNA substrate, wrapping completely around the DNA [4]. Topotecan stabilizes the covalent topoisomerase 1 – DNA complex. The stabilized breaks are fully reversible and non-lethal [3, 5]. In models of camptothecin and its derivatives, they are proposed to be stacked between DNA bases.

Topotecan is currently in widespread use in clinical practice in the treatment of small cell lung cancer and ovarian cancer. Topotecan is known to increase progression-free survival in SCLC and ovarian cancer and many new topotecan studies are underway [6–8]. A better knowledge of molecular and biochemical mechanisms of actions of this drug is crucial to design more optimal combination treatments with topotecan, and to better select the patients who can benefit from topotecan treatment.

HIF-1 is a master regulator of cellular and developmental O_2 homeostasis and the cellular response to hypoxia [9, 10]. The HIF-1 transcription factor consists of two subunits, HIF-1α and HIF-1β. HIF-1β is constitutively expressed, while the amount of HIF-1α protein is

regulated by hypoxia, through O_2-dependent prolyl hydroxylation, which targets the protein for degradation [11, 12]. HIF-1 is a transcription factor for a number of genes relevant to adaptation, and it also interacts with other transcription factors [10]. The genes regulated by HIF-1 include the angiogenic growth factor VEGF. HIF-1α is upregulated in many human tumors and inhibitors of HIF-1α are underway and are currently being tested for their efficacy as anticancer therapeutics [13, 14].

The aim of this study was to evaluate the role of hypoxia as a modifier of the response to topotecan, and see if topotecan treatment delivered under moderately, i.e., clinically feasible, hypoxic conditions results in differences in cell survival and tumor growth.

Materials and methods

Cell lines

The mouse Lewis lung carcinoma cell line was routinely maintained in RPMI-1640 with 10% FCS. The human small cell lung cancer line DMS 273 was grown in Waymouth's medium with 10% FCS and the human glioblastoma line U87 was maintained in Eagle's MEM with 10% FCS. All cell lines were propagated at 37°C in a humidified atmosphere containing 5% CO_2 and 21% O_2.

Animals and tumors

Male 7-week-old athymic nude mice (NMRI-*nu*/*nu*) were used for experiments with the two human cell lines DMS 273 and U87 and C57bl/6 mice were used for experiments with Lewis lung carcinoma. All mice were obtained from Taconic (Ry, Denmark). The mice were kept in individually ventilated Sealsafe cages (Scanbur, Koege, Denmark). They received sterile food pellets and water ad libitum. Institutional guidelines for animal welfare and experimental conduct were followed.

U87 and DMS273 xenografts were established by subcutaneous injection of tumor cells, and maintained by serial transplantation. Prior to transplantation, the mice were anaesthetized by a subcutaneous injection of ketamine (10 mg/kg) and xylazine (1 mg/kg) in 0.9% NaCl solution. Through a 1-cm incision in the dorsal skin, 1-mm^3 tumor blocks were subcutaneously implanted into the right flank. For experiments with LLC, the cells were propagated in vitro and 10^6 cells were injected subcutaneously in the right flank of each mouse.

Tumors were measured 5–7 days a week during tumor growth, by two orthogonal diameters d_1 and d_2. Tumor volume was calculated according to the equation: $V = \pi/6(d_1 \times d_2)^{3/2} \times k$, where k is an empirical constant = 0.67[15].

Treatment

Topotecan (SmithKline Beecham) was purchased from a local distributor. Mice received doses of 20 mg/kg or 15 mg/kg i.p. For cell culture experiments, other than clonogenic survival assay, the drug was dissolved in sterile water to a concentration of 1 mg/ml and further diluted in growth medium to the concentrations required for each experiment. Cisplatin (Sigma) was dissolved in sterile saline, and mice were treated with 6 mg/kg i.p.

Hypoxic conditions

Cell cultures were incubated in an $InVivo_2400$ Hypoxia Workstation (Ruskinn) in a humidified atmosphere containing 5% CO_2 and O_2 from 0.1% to 0.7% at 37°C. Cell culture medium and cells for protein analysis were harvested while in the hypoxia workstation, and were not re-oxygenated before harvesting.

Mice were placed at 10% oxygen in a custom build chamber for 24 h (tumors collected for VEGF measurements) and 6 h or 72 h for growth experiments. The 10% O_2 was well tolerated.

Clonogenic survival assay

The clonogenic survival of Lewis Lung Carcinoma cells during continuous topotecan treatment was assessed in RPMI-1640 containing 10% FCS and penicillin/streptomycin (50 U/ml) at 37°C in a humidified atmosphere of 5% CO_2 and 0.1% or 21% O_2. Cells were counted and plated in 10 ml growth medium onto 100-mm dishes and incubated over night for attachment. In dishes subjected to 0.1% O_2, 1×10^4 cells were added, while 1×10^3 cells were added in each dish subjected to 21% O_2. Topotecan was dissolved in DMSO at different concentrations so equal volumes of drug were added to each plate (0.1% DMSO). Topotecan was added to the cells at the indicated doses prior to a 24-h incubation at 0.1% or 21% O_2. After 24 h, all plates were incubated for 10–12 days at 21% O_2, colonies were fixed and stained in 0.4% methylene blue in methanol (Sigma, Copenhagen) and colonies with more than 50 cells were counted. Clonogenic survival was calculated by dividing the mean number of colonies for each treatment by the mean number of colonies on control plates treated with 0.1% DMSO. All treatments were performed in duplicate and the experiments were repeated independently three times. In a separate normoxic set of experiments, the clonogenic survival of cell cultures treated for 24 h with topotecan at pH 7.2 and pH 6.7 was compared to control cells. After 24 h the medium in both treatment groups were changed to normal, topotecan free medium and all plates were incubated for 10–12 days before fixing and staining as described above.

MTT assay

The sensitivity to topotecan of LLC cells at the doses used to down regulate the VEGF secretion was analyzed by a MTT proliferation assay. Cells were plated in 96-well plates at a density of 1×10^4 cells/well and allowed to attach O/N. The RPMI-1640 culture medium containing 10% FCS was changed to RPMI-1640 containing 10% FCS and different doses of topotecan from 0 nM to 270 nM, in a humidified atmosphere of 5% CO_2 and 0.7% or 21% O_2. After treatment for 24 h, medium was harvested for ELISA measuring of VEGF as described above. Then 20 µl (5 mg/ml) MTT [3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, Sigma] was added to each well and then incubated for 4 h. After this, 100 µl 10% SDS/0.01 M HCl solution was added and incubated O/N. Plates were read with absorbance at wavelength at 570 nm and the background absorbance at 690 nm.

ELISA

Cell culture medium was briefly spun, a proteinase inhibitor cocktail, Complete (Roche, Hvidovre, Denmark) was added, and the medium was frozen at −80°C. LLC tumor blocks were obtained 24 h after start of topotecan and hypoxia treatment. and homogenized with five short bursts on a Vibra Cell 50 (Sonics and Materials Inc.). The protein concentration was determined with a BCA Protein Assay (Pierce Inc.). Blood samples from mice with LLC were collected from the heart of anaesthetized mice. 3.8 µmol EDTA was added per ml blood, followed by 30 min centrifugation at 3,000 rpm. Plasma was collected and stored at −80°C. The VEGF protein content in medium, tumor protein and EDTA stabilized plasma was determined using human and mouse VEGF Quantikine immunoassay kits (R&D Systems, Abingdon, U K) according to the manufactures instructions.

Western blot analysis

Hypoxic cell cultures were harvested while under hypoxic exposure. Cell lysates were sonicated by ultrasound using a Vibra Cell 50 (Sonics and Materials, Danbury CT, USA).

The protein concentration was determined with a BCA Protein Assay (Pierce Inc., Roedovre, Denmark) and 10/20/40 µg of protein were separated on 1.0 mm NuPage 3-8% Tris–acetate gels (Invitrogen, Taastrup, Denmark) and transferred onto nitrocellulose membranes (Invitrogen, Taastrup, Denmark) by semi-dry blotting.

Membranes were blocked in Tris-buffered saline pH 7.4, 10% skim milk powder, 0.1% Tween-20, and washed in Tris-buffered saline pH 7.4. Incubation with primary and secondary antibodies for 1–16 h was followed by wash in Tris-buffered saline, pH 7.4, and 0.1% Tween-20. The protein expression was visualized with ECL + Plus or ECL-Advance (Amersham, Hoersholm, Denmark) and quantified by chemifluorescence scanning on a STORM 840 (Molecular Dynamics Inc Chesham, U K). α-tubulin expression was used for loading control. The following antibodies and concentrations were used: Monoclonal mouse-anti-human-VEGF (1 µg/ml), (BD Pharmingen, Broendby, Denmark), monoclonal mouse-anti-HIF-1α (1 µg/ml), (BD Transduction Laboratories, Broendby, Denmark), polyclonal chicken-anti-mouseHIF-1α, (1:400), (kindly provided by Max Gassmann, University of Zurich). Polyclonal goat-anti-mouseVEGF (R&D Systems, Abingdon, U K), Monoclonal mouse-anti-human Glut-1 (F18) was kindly provided by Novo Nordisk, Denmark. Monoclonal mouse-anti-α-tubulin (1:10.000) was obtained from Sigma, (Vallensbaek Strand, Denmark). Horseradish peroxidase conjugated polyclonal goat-anti-mouse-immunoglobulin (1:3,000 and 1:5,000), and horseradish peroxidase conjugated polyclonal rabbit-anti-goat-immunoglobulin (1:2,000) was obtained from DAKO (Glostrup, Denmark). Horseradish peroxidase conjugated polyclonal donkey-anti-chicken-immunoglobulin (1:5,000) was obtained from Jackson ImmunoResearch (TriChem, Frederikssund, Denmark).

Results

Tumor growth inhibitory effects of topotecan is increased by a hypoxic environment

We evaluated the growth of three different tumor lines on mice, Lewis lung carcinoma and the two human cancer lines DMS273 (small cell lung cancer) and U87 (glioblastoma multiforme) upon topotecan-treatment with or without exposure of the mice to a hypoxic environment. In all three tumor lines, we found an increase in the growth inhibitory effect of topotecan, when the treatment was combined with a hypoxic environment (10% O_2) for 72 h. Seventy-two hours hypoxia exposure time was chosen in order to ensure that the mice were hypoxic throughout the exposure time for topotecan. The time for subcutaneous LLC tumors to reach two volume doublings [V(pretreatment)×4] was significantly increased after topotecan treatment under hypoxia, compared with topotecan alone (Table 1). This was also the case when the time in hypoxic environment was reduced to 6 h (Table 1). 20 mg/kg was well-tolerated by C57black mice with LLC tumors, but was toxic in nude NMRI mice. In an experiment with U87 tumors on NMRI mice, seven and eight of the 12-mice groups treated with topotecan or topotecan and hypoxia died within few days after the end of treatment. Still, the mean volume change $\Delta V = [(V_{72--h} - V_0)/V_0]$ in U87 tumors treated by a single dose of 20 mg/kg topotecan under hypoxia was significantly lower than in tumors

Table 1 Hypoxic exposure (72 and 6 h) significantly increased the inhibitory effect of topotecan on tumor growth, while hypoxic exposure did not change the effect of cisplatin

	Time until two tumor volume doublings				
	n	Mean days	SD	Logrank test versus controls	Logrank test versus topotecan
Experiment 1					
Controls	*10*	5.7	2.1		
Topotecan 20 *mg/kg* ×1	*6*	6.7	1.0	NS	
Hypoxia *72 h at 10%O_2*	*9*	6.3	1.7	NS	
Topotecan 20 *mg/kg* ×1 + Hypoxia *72 h at 10%O_2*	*9*	8.8	2.1	$P=0.004$	$P=0.005$
Experiment 2					
Controls	*10*	4.0	0.9		
Topotecan *20 mg/kg* ×1	*12*	6.0	1.2	$P=0.001$	
Hypoxia *6 h at 10%O_2*	*10*	4.5	1.4	NS	
Topotecan *20 mg/kg* ×1 + Hypoxia *6 h at 10%O_2*	*12*	7.1	1.3	$P<0.001$	$P=0.04$
Experiment 3					
Controls	13	6.1			
Cisplatin *6 mg/kg* ×1	12	8.3		$P=0.01$	
Hypoxia *6 h at 10%O_2*	12	5.1		NS	
Cisplatin *6 mg/kg* ×1 + Hypoxia *6 h at 10%O_2*	12	8.7		$P=0.01$	NS

Growth characteristics of LLC tumors after topotecan or cisplatin with or without hypoxic environment.For each individual tumor the time from treatment start until the first measurement of a volume of four times the volume at treatment start (two volume doublings) was recorded

treated with topotecan under normoxia ($P=0.000$, $n=12$ in each group, *t* test). Due to the toxicity in NMRI mice, we reduced the dose to 15 mg/kg once a week, and DMS273 showed a significantly reduced growth after two series of 15 mg/kg topotecan combined with 72 h hypoxia with an interval of 7 days between series (Fig. 1). In all three tumor lines, the growth inhibition was significantly higher in tumors treated with topotecan under hypoxia than in tumors treated with topotecan under normoxic conditions. In contrast, the effect of another chemotherapeutic drug, cisplatin, was not altered by 10% hypoxic environment (Table 1,- Fig. 2). Cisplatin is a platinum compound that elicits its antitumor effect through DNA cross-linking. Hypoxic environment alone did not significantly change the tumor growth (Table 1).

Hypoxia increases topotecan sensitivity of Lewis lung carcinoma cells

To test the hypothesis that hypoxia is involved in topotecan cytotoxicity, we measured the in vitro topotecan sensitivity of Lewis lung carcinoma cells during hypoxic and normoxic growth conditions (Fig. 3A). At hypoxic growth conditions we observed a three-fold higher cytotoxic effect of topotecan ($IC_{50}=11.1$) compared to normoxia ($IC_{50}=36.2$). $P=0.01$ (*t* test).

The finding of an increased effect under hypoxia was further confirmed by MTT cytotoxicity assay, where a 24-h topotecan treatment at 0.7% O_2 significantly reduced the number of viable cells, while no effect was observed under normoxic growth (Fig. 3B).

In a different model system where in vitro topotecan treated cells were implanted on mice after treatment, it was previously suggested that topotecan was more effective if pH was low in the treatment medium[16]. We therefore measured the pH in the RPMI-1640 treatment medium at 24 h exposure to 0.1% oxygen, and found a mean pH value of 6.7 with little variation in several samples. The normoxic pH was 7.2. In a subsequent experiment, the clonogenic survival of normoxic LLC cells treated with topotecan at pH 7.2 was compared to topotecan treatment at pH 6.7. No difference in clonogenic survival was observed (Fig. 3C).

Topotecan reduces the HIF-1α and VEGF expression

HIF-1 is the primary transcription factor for hypoxic upregulation of VEGF and Glut-1 [10, 17]. It has previously been reported [1] that topotecan treatment downregulates HIF-1α in U251 glioma cells. By immunoblotting, we measured HIF-1α, Glut-1 and VEGF and found that intracellular HIF-1α, Glut-1 and VEGF protein levels were downregulated by topotecan in our three cells lines as well (Fig. 4). Quantification of immunoblots from three experiments shoved that the intracellular VEGF level was significantly decreased at 60 nM and 120 nM ($P<0.02$, t-test), compared with controls.

Since VEGF is a secreted protein, we focused on the secreted fraction, and measured the VEGF protein levels in cell culture medium by ELISA. In both LLC, U87 and DMS273, we found that the hypoxic upregulation of VEGF secretion was inhibited by topotecan at doses that did not affect the normoxic levels (Fig. 5). In a separate experiment, 20 mice with LLC were treated with topotecan 20 mg/kg or vehicle, and / or hypoxic environment for 24 h, in parallel with the growth inhibition

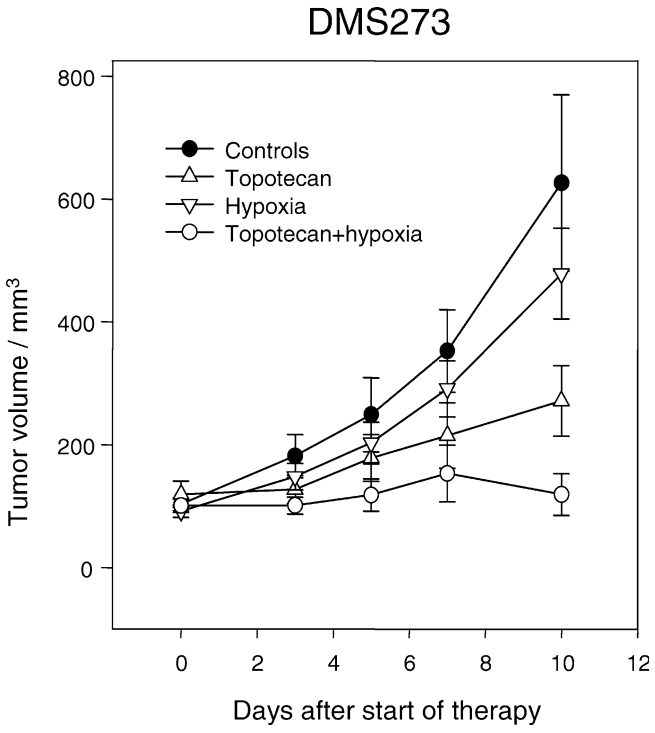

Fig. 1 Hypoxic environment enhanced the effect of topotecan treatment on subcutaneous DMS273 tumor volume. Mice with DMS 273 tumors were included in the experiment at a mean tumor volume of 100 mm^3. The mice were treated with topotecan 15 mg/kg i.p., or saline i.p. Immediately after injection, the mice were placed in a chamber with 10% oxygen, or in normoxic environment. After 3 days, mice were taken out of the chamber and the tumors were measured again. This treatment with either topotecan or saline and / or hypoxic environment was repeated 1 week after the first treatment round, i.e., topotecan on day 0 and day 7, hypoxic exposure 72 h from day 0 and from day 7. The tumor volume on day 10 was compared. The effect on tumor volume of combined topotecan and hypoxia was significantly different from controls ($P = 0.005$) and topotecan alone, ($P = 0.04$, t test). Hypoxia alone did not significantly affect tumor growth compared with controls, ($P = 0.4$, t-test). The dose of topotecan was sufficient to cause reduction in tumor growth under normoxia as well ($p = 0.04$, t test, controls vs. topotecan alone). $n = 9$–11 in each group. *Bars* Standard deviation

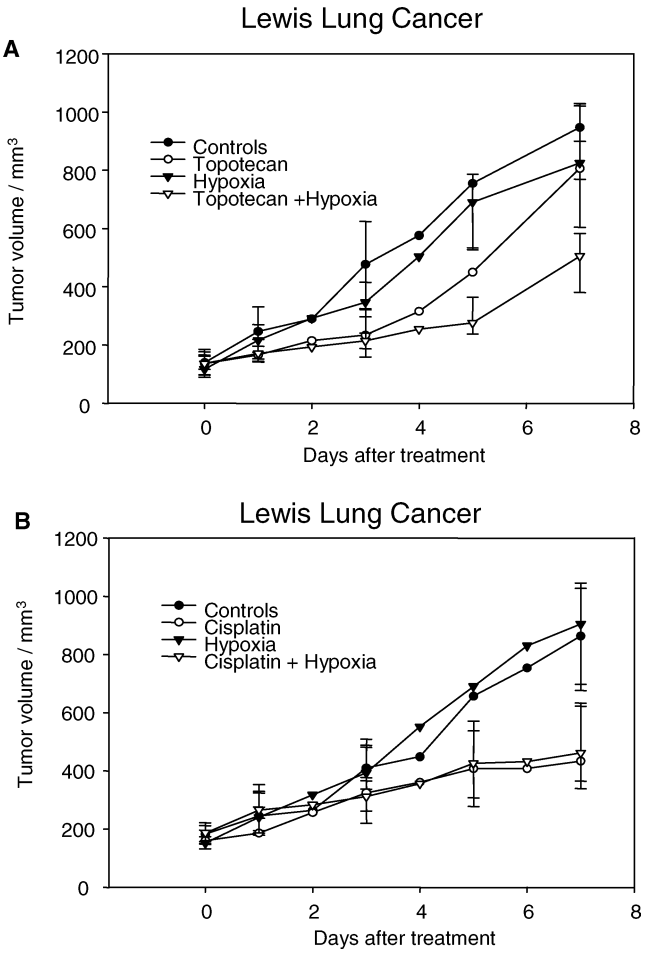

Fig. 2 Hypoxic environment enhanced the growth inhibitory effect of topotecan on Lewis lung cancer, while cisplatin growth inhibition was unaffected. **A** C57/bl mice with subcutaneous LLC tumors were treated with Topotecan 20 mg/kg i.p., immediately followed by 6 h exposure to hypoxic environment (10% oxygen), or normoxic environment. Saline-treated mice were exposed to 6 h hypoxic environment, or normoxia (controls). The growth inhibitory effect of topotecan was significantly enhanced by hypoxic environment. **B** In a parallel control experiment, growth inhibition induced by cisplatin 6 mg/kg i.p., was unaffected by hypoxic environment. $n = 12$–13 in each group

experiment. These mice were sacrificed at the end of 24 h hypoxic exposure, and blood and tumor samples were harvested. The intratumoral VEGF content was significantly lower in topotecan treated mice, both in normoxic and hypoxic environment (Fig. 6). Tumors from mice in hypoxic environment did not show an upregulation in VEGF levels in this experiment. No difference in plasma VEGF levels were observed between groups (data not shown).

Discussion

The in vitro finding of increased cytotoxicity and reduced clonogenic survival of tumor cells treated with topotecan under hypoxic circumstances confirm that a part of the effect of topotecan is related to a decrease in hypoxia tolerance of tumor cells. Our experiments also shows that the increased effect under hypoxic conditions is not an effect of the reduction in pH, since lowering the pH under normoxic conditions did not increase the effect of topotecan. Topotecan reduces the hypoxic upregulation of HIF-1α, and reduces the HIF-1 transcriptional response to hypoxia in U251 cells[1]. Our studies show that this is also the case in three other cancer lines, and that this effect does indeed affect the survival of the cells under hypoxia (Fig. 3). Since HIF-1 is the major transcriptional regulator of the response to hypoxia, our in vitro experiments confirm that the effect of HIF-1 inhibition is not due to inhibition of angiogenesis alone, and stresses the fact that other effects of HIF-1 are important for cells to survive under hypoxic circumstances.

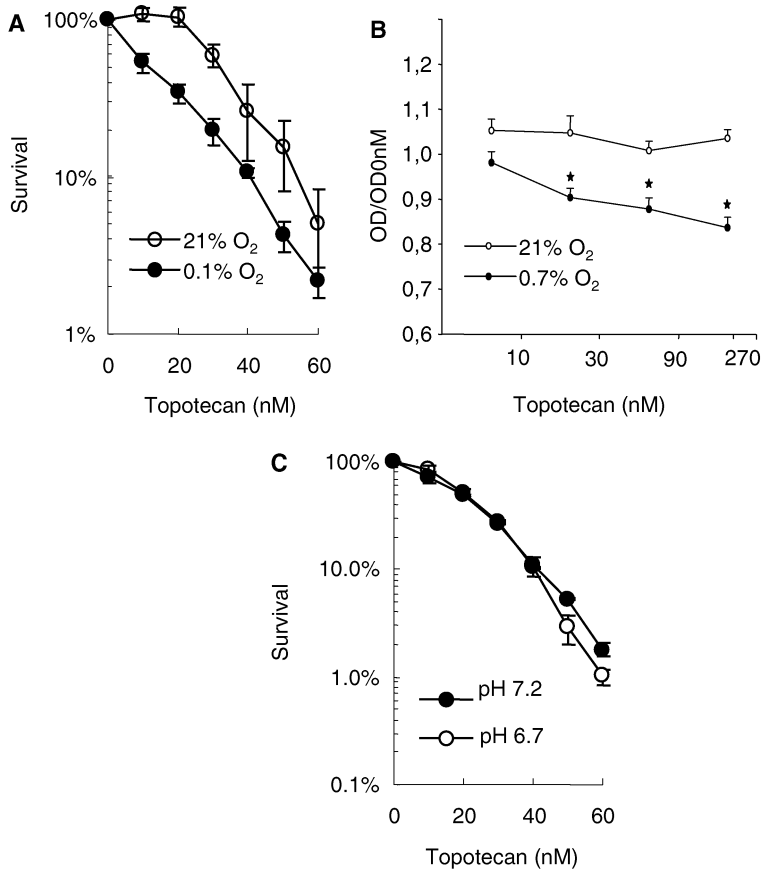

Fig. 3 Hypoxic growth conditions sensitize Lewis lung carcinoma cells to the toxic effects of topotecan. **A** Clonogenic survival assay in Lewis lung carcinoma cells upon continuous topotecan treatments under hypoxic (0.1% O_2) or normoxic (21% O_2) growth conditions. Mean fraction of surviving colonies, relative to DMSO treated control plates. The mean (*symbols*) and standard deviation (*error bars*) of three independent experiments are presented. **B** MTT cytotoxicity assay. Topotecan for 24 h at 0.7% O_2 significantly reduced the number of viable LLC cells at doses from 30 nM ($P < 0.003$, *t* test). 10 nM–270 nM did not affect the viability of normoxic LLC cells. $n = 8$ in each group. *Bars* Standard deviation. **C** Clonogenic survival assay in Lewis lung carcinoma cells upon 24 h topotecan treatment in pH 7.2 or pH 6.7. Mean fraction of surviving colonies, relative to DMSO treated control plates. The difference in pH did not change the effect of topotecan

The actual changes in levels of oxygen inside the tumors were not measured in these investigations, but it has been shown previously that changes in the oxygen content of breathing air can change the oxygen levels in tumors[18, 19]. It is well known that most solid tumors have areas of chronic and acute hypoxia as well as necrotic areas due to a chaotic and insufficient vasculature and blood supply. It is therefore not surprising that a reduction in oxygen content in the breathing air did not result in an increase in the VEGF content in tumor samples, which probably include areas with sustained hypoxia refractory to the oxygen status of the blood. The contribution of VEGF from cells in areas where hypoxia is a derived effect of the low oxygen content in the blood, might disappear in the large pool of VEGF from tumor segments with chronic hypoxia. Since topotecan reduced the levels of VEGF in both hypoxic and normoxic tumors, HIF-1α must be upregulated in all tumors independent of the oxygen content of the breathing gas. On this background, the increase in growth inhibitory effect of topotecan under hypoxia is impressive and might reflect that the most actively dividing cells in a tumor are normoxic or only moderately hypoxic and that they are especially sensitive to a decrease in the blood oxygen content.

VEGF is an important angiogenic factor and also a survival factor for endothelial cells[20]. Low-dose topotecan has been shown to have antiangiogenic effect in

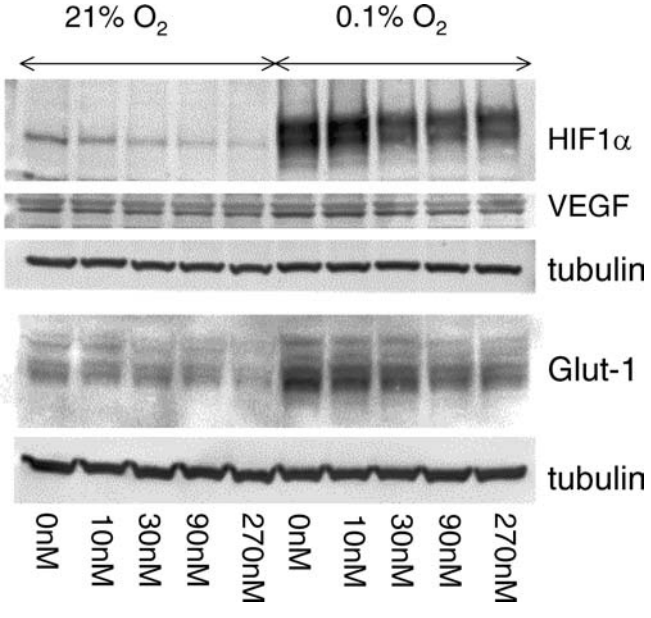

Fig. 4 The intracellular levels of HIF 1α, VEGF and Glut-1 were dose-dependently downregulated by topotecan. The downregulation of HIF-1α and the two HIF-1 transcriptional regulated proteins VEGF and GLUT-1 was seen under both normoxic and hypoxic conditions, although the HIF-1α expressionwas very low under normoxic conditions. Incubation time: 24 h. DMS273 representative immunoblots from several independent in vitro experiments

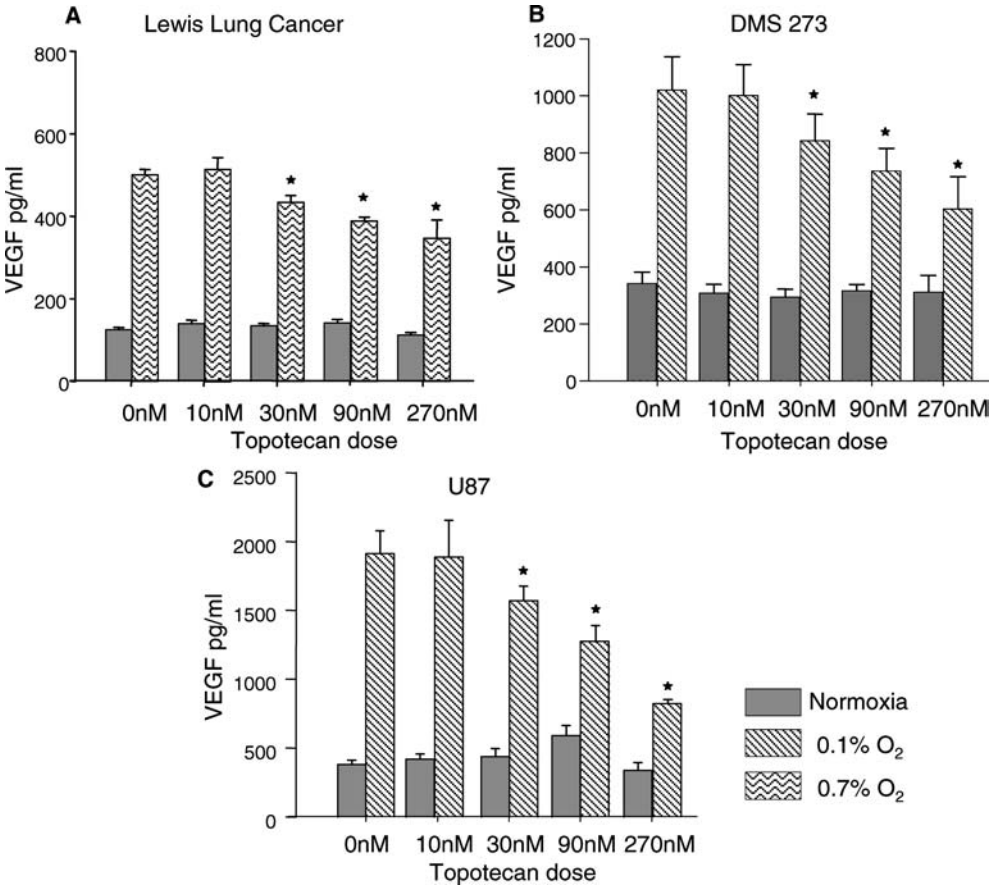

Fig. 5 VEGF secretion was reduced by topotecan in hypoxic cell cultures. Topotecan significantly reduced the hypoxic induction of VEGF secretion to the cell culture medium at doses from 30 nM in both LLC DMS273 and U87. No change in VEGF secretion was seen in normoxic cell cultures. **A** LLC cells $n=8$ in each treatment group **B** DMS273 cells. $n=8$ in each treatment group. **C** U87cells. $n=4$ in each treatment group. *Bars* Standard deviation. *Asterixes* $P<0.01$ compared with controls (*t* test)

both a sponge disc in vivo angiogenesis assay[21] and in the classical mouse cornea model[22]. In both these assays basic fibroblast growth factor, bFGF was used to induce angiogenesis, and bFGF acts synergistically with VEGF in angiogenesis[23]. So the antiangiogenic effect in these two studies might very well be explained by the downregulation of VEGF.

In conclusion, our study shows that topotecan is more potent in hypoxic tumor cells than in normoxic cells, and that the growth inhibition in tumors is greater when topotecan is combined with hypoxia. This has potential clinical implications. First, the knowledge could be used to select patients with very hypoxic tumors who might benefit particularly from topotecan treatment. Second, administration of topotecan in a moderately hypoxic

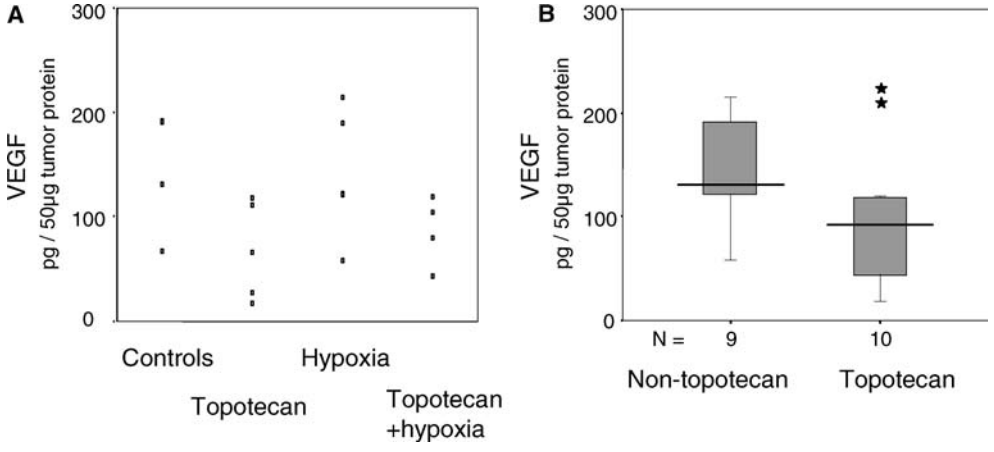

Fig. 6 VEGF in tumors was decreased by topotecan treatment. **A** 4-5 LLC tumors were treated with topotecan 20 mg/kg i.p. or saline followed by exposure to hypoxic environment (10% O_2) or normoxia for 24 h. Immediately after opening of the hypoxic chamber, the mice were anaesthetized and blood and tumors were harvested. **B** Topotecan treated (with or without hypoxia) compared with non-topotecan treated (with or without hypoxia). Topotecan treatment significantly reduced the level of VEGF protein in LLC tumors ($P=0.009$, *t* test)

environment calls for clinical testing. Finally, the anti-HIF effect of topotecan should be taken into consideration when combining topotecan and other drugs. Based on our studies here, we expect that topotecan will act synergistically in combination with drugs that evokes acute hypoxia, e.g., vascular damaging agents.

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