Low-Dose Metronomic Daily Cyclophosphamide and Weekly Tirapazamine: A Well-Tolerated Combination Regimen with Enhanced Efficacy That Exploits Tumor Hypoxia

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Abstract
The recent clinical successes of antiangiogenic drug-based therapies have also served to highlight the problem of acquired resistance because, similar to other types of cancer therapy, tumors that initially respond eventually stop doing so. Consequently, strategies designed to delay resistance or treat resistant subpopulations when they arise have assumed considerable importance. This requires a better understanding of the various possible mechanisms for resistance. In this regard, reduced oxygenation is thought to be a key mediator of the antitumor effects of antiangiogenic therapies; accordingly, increased hypoxia tolerance of the tumor cells presents a potential mechanism of resistance. However, hypoxia can also be exploited therapeutically through the use of hypoxic cell cytotoxins, such as tirapazamine. With this in mind, we measured the oxygenation of PC-3 human prostate cancer xenografts subjected to chronic low-dose metronomic (LDM) antiangiogenic chemotherapy using cyclophosphamide given through the drinking water. We found that LDM cyclophosphamide impairs the oxygenation of PC-3 xenografts even during relapse, coinciding with reduced microvessel density. Combination of LDM cyclophosphamide with tirapazamine results in significantly improved tumor control in the PC-3, HT-29 colon adenocarcinoma, and MDA-MB-231 breast cancer human xenograft models without having a negative effect on the favorable toxicity profile of LDM cyclophosphamide. These results provide further evidence that reduced vascular dependence/increased hypoxia tolerance may be a basis for eventual resistance of tumors exposed to long-term LDM chemotherapy. (Cancer Res 2006; 66(3): 1664-74)

Introduction
The concept of antiangiogenic therapy for cancer was first outlined >30 years ago (1). After several decades of promising preclinical studies, it was recently clinically validated with the report of increased median and progression-free survival in a large randomized phase III trial involving bevacizumab (Avastin), a humanized anti–vascular endothelial growth factor (VEGF) monoclonal antibody, combined with irinotecan, 5-fluorouracil, and leucovorin chemotherapy for the first-line treatment of metastatic colorectal cancer (2). Subsequently, the benefit of combining bevacizumab with various standard chemotherapy regimens was further documented in randomized phase III trials for second-line treatment of metastatic colorectal cancer and first-line treatment of metastatic breast or late-stage nonsquamous non–small cell lung cancer (3). Moreover, multitargeted, antiangiogenic small-molecule receptor tyrosine kinase inhibitors, notably sunitinib (Sutent, SU11248) and sorafenib (Nexavar, BAY 43-9006), have shown very promising results as monotherapies in the treatment of renal cell carcinoma (3).

Besides these targeted therapies, another antiangiogenic treatment strategy, low-dose metronomic (LDM) chemotherapy, has shown promising activity in many preclinical and several phase I/II human studies involving various tumor types, such as metastatic breast, prostate, and recurrent ovarian cancer (4, 5). LDM is the administration of comparatively low doses of cytotoxic drugs on a frequent or continuous schedule, with no extended breaks. As opposed to conventional maximum tolerated dose (MTD) chemotherapy, LDM is thought to act mainly via antiangiogenic mechanisms (6) and, in addition, has a very favorable toxicity profile (4, 7). Moreover, LDM potentially is very cost-efficient, especially when using off-patent chemotherapeutic drugs, such as cyclophosphamide and methotrexate (8), a conceivably important additional benefit in the light of the rapidly escalating costs of new cancer drugs and therapies, an economic issue that is taking on increasing urgency (9).

Although, in theory, some antiangiogenic therapies are expected to be less prone to the rapid development of acquired resistance (10), with few exceptions (6, 11), the therapeutic benefit of LDM-based and antiangiogenic strategies in general is subject to eventual resistance (2, 12). The basis for acquired (and intrinsic) resistance to antiangiogenic treatment regimens is currently largely unknown. Some possibilities include angiogenic growth factor redundancy when one specific growth factor is targeted, compensatory responses, such as vascular remodeling, relative angiogenesis-independent tumor growth, and reduced vascular dependence/increased hypoxia resistance of the tumor cell population (13–16).

Information regarding (epi)genetic and physiologic consequences of antiangiogenic therapies is similarly limited and experimental results are sometimes conflicting (17, 18). This is particularly true with respect to the effect on intratumoral oxygen tension, which is of special interest and importance for several reasons. Besides low pH, reduced nutrient availability, and high interstitial fluid pressure, reduced oxygenation is a hallmark of the tumor microenvironment (19). Additionally, hypoxia is a well-known mediator of resistance to radiation and cytotoxic drugs and contributes to tumor progression as well as metastasis (20). Given that the antitumor effects of antiangiogenic therapies are believed
to result primarily from increased hypoxia (or even anoxia; ref. 17), such therapies might eventually lead to more aggressive tumor regrowth after initial regression via hypoxia-driven mechanisms. In addition, hypoxia may contribute to resistance to antiangiogenic cancer therapy (21, 22).

On the other hand, tumor hypoxia can itself be exploited by various therapeutic strategies. For example, the pO2 differential between the tumor microenvironment and healthy tissues can be therapeutically "targeted" using prodrugs activated under conditions of reduced oxygen tension (19). Tirapazamine is clinically the most advanced compound of a group of agents called hypoxic cytotoxins that are reduced ("activated") under hypoxic conditions resulting (in the case of tirapazamine) in metabolites and degradation products causing topoisomerase II poisoning and DNA damage, particularly double-strand breaks (19). Interestingly, tirapazamine also exhibits "accidental" antiangiogenic (23, 24) and vascular targeting effects (25), potentially targets hypoxic tumor endothelial cells (26), and affects tumor blood flow by virtue of its vasoactive properties (27). However, the antivascular effects might be context dependent. For example, Cao et al. showed recently that tirapazamine does not significantly impair incipient, hypoxia-independent angiogenesis (28). Tirapazamine has already successfully been combined with vascular disrupting agents (29) and antiangiogenic drugs, such as TNP-470 (30), albeit only for short treatment protocols.

Because we reported recently that tumor cell hypoxia resistance may contribute to resistance to LDM-based treatment regimens (15), it occurred to us that this form of resistance might be delayed and/or exploited by combining LDM with tirapazamine. Here, we present the first detailed study of the effect of LDM cyclophosphamide, clinically the most advanced "metronomic" drug at the present time (4), on the oxygenation status of PC-3 human prostate cancer xenografts. The PC-3 model was chosen because of its very reproducible growth pattern during long-term LDM cyclophosphamide therapy, where a delayed regression phase is followed by stable disease and finally by eventual progression while on therapy (31). Moreover, the degree of hypoxia and its effect is well documented in prostate carcinomas (32). We show that LDM cyclophosphamide significantly decreases microvessel density and oxygenation in PC-3 xenografts, effects that are sustained during progression, and confirm the antiangiogenic effects of tirapazamine. When used in combination with LDM cyclophosphamide, tirapazamine impairs the growth of PC-3, HT-29 colon adenocarcinoma, and MDA-MB-231 breast cancer xenografts despite showing very minor activity as a cytotoxic agent alone (as outlined below). After three passages under conventional tissue culture conditions, the homogenous tumor cell populations were frozen until further use.

**Mice and tumor xenograft models.** All animal procedures were done in accordance with institutional and national guidelines. Mice were allowed to acclimatize and mature before implantation of Matrigel or tumor cells. Female BALB/cj mice (9 weeks old) used for Matrigel plug perfusion were purchased from The Jackson Laboratory (Bar Harbor, ME). Bovine aortic endothelial cells were a gift of Dr. J. Folkman (Children’s Hospital, Boston, MA). PC-3 and bovine aortic endothelial cells were maintained in DMEM and MDA-MB-231 and HT-29 cells were maintained in RPMI 1640, both media supplemented with 5% FCS in a humidified atmosphere of 5% CO2 at 37°C.

**Treatment regimens.** Continuous LDM cyclophosphamide (20 mg/kg/d) was given daily with no breaks in the drinking water according to Man et al. (31). Tirapazamine solution (1 mg/mL) was injected i.p. weekly to achieve a dose of 25 mg/kg/wk (0.14 mg/mL/kg/wk). Control animals received corresponding amounts of 0.9% normal saline orally or i.p. as required, the only nonmedicinal ingredient in the cyclophosphamide powder and diluent of tirapazamine.

**Matrigel plug perfusion assay.** The Matrigel plug perfusion assay was done as described (7). Briefly, 0.5 mL Matrigel supplemented with 500 ng/mL bFGF was injected s.c. on day 0 into the flanks of female BALB/cj mice. Treatment was initiated after 72 hours. After 10 days, mice were injected i.v. with 0.2 mL of 25 mg/mL FITC-dextran, plasma was collected, and Matrigel plugs were removed, digested with Dispase (at 37°C overnight), and homogenized. Fluorescence readings were obtained using a FL600 Fluorescence Plate Reader (Bio-Tek Instruments, Winooski, VT). The level of the angiogenic response was assessed as ratio of Matrigel plug fluorescence to plasma fluorescence.

**EF5 immunofluorescence and flow cytometric analysis.** EF5 stock solution (10 mM/l) prepared in 0.9% normal saline was injected i.p. (10 /L/g body weight) 3 hours before the mice were sacrificed. For immunofluorescence analysis, 6-μm frozen tumor sections were fixed with 4% paraformaldehyde (pH 7.0) for 1 hour at 4°C. After rinsing (PBS) and blocking overnight at 4°C [5% mouse serum, 20% milk (10% skimmed milk powder in PBS), 75% antibody carrier PBS (PBS with 0.3% Tween 20 and 1.5% bovine serum albumin)], sections were incubated for 2 hours at room temperature with ELK3-51 antibody (75 μg/mL). Next, sections were rinsed thrice (PBS) and incubated with secondary Texas Red–conjugated F(ab)2.
goat anti-mouse IgG (7.5 μg/mL) for 15 minutes followed by four rinses (PBStt). Nuclei were counterstained with DAPI (1 μg/mL in PBS) for 2 minutes. Images were acquired with a Carl Zeiss Axioplan 2 microscope (fluorescence filters: 359 nm excitation/461 nm emission for DAPI and 555 nm excitation/620 nm emission for Texas Red) and a Zeiss Axioctam camera using AxioVision 3.0 software at ×100 magnification and then exported to Adobe Photoshop 6.0 software (Adobe Systems, Inc., San Jose, CA). For quantification, the mean fluorescence plus 2 SDs of three control tumors (mice injected with 0.9% saline) was set as the hypoxia threshold. The mean and median fluorescence intensity of the various treatment groups were expressed as mean ± SD (three to six fields per tumor, excluding necrotic areas; n = 3 tumors per group).

For flow cytometric analysis, tumors were mechanically and enzymatically (using collagenase type III at 4 mg/mL, collagenase type IV at 2 mg/mL, and hyaluronidase at 2 mg/mL in serum-free DMEM) disrupted to a single-cell suspension, fixed in ice-cold 4% paraformaldehyde for 1 hour, permeabilized with PBStt, and stored at −70°C in 25% glycerol. After blocking overnight at 4°C (using 4% normal mouse serum and 16% milk in PBStt), 1 × 105 cells were incubated with ELK3-51 antibody (75 μg/mL) at room temperature for 3 hours, washed (2 × PBStt, 1 × PBS), and resuspended in 20 μg/mL 7-AAD in PBS. Cells were analyzed on a FACSCalibur cell analyzer (Becton Dickinson, Franklin Lakes, NJ) with CellQuest Pro acquisition software (Becton Dickinson, Mountain View, CA). Viable tumor cells were gated based on forward and backward scatter and DNA content (7-AAD). The percentage of viable hypoxic cells as well as mean and median fluorescence intensity (n = 3 per treatment group) were calculated using a threshold defined by the mean fluorescence plus 2 SDs of control cells from three tumors not exposed to EF5, and the data were expressed as mean ± SE.

pO2 measurements. Intratumoral O2 tension was measured using a polarographic oxygen electrode (Eppendorf, Germany, model 6650). The “Histograph” was calibrated according to the manufacturer’s instructions and set to a step length of 1.0 mm with a retraction step of 0.3 mm. An electrocardiogram patch was attached to the abdominal wall as an anode. The mice were anesthetized with enflurane, an inhalation anesthetic that does not interfere with oxygen electrode measurements (33). Body temperature was maintained with an external heating device. Some rare measurements less than −0.5 mm Hg were excluded from the analysis.

Proliferation assay. Cells (n = 4,000 per well for tumor cell lines or n = 1,500 per well for bovine aortic endothelial cells) were plated in 96-well plates in 100 μL medium containing 10% FCS. The next day, 100 μL tirapazamine (LDM cyclophosphamide) medium was added to each well, and the cells were incubated under either normoxic (19.6% O2, 5% CO2, 75.4% N2) or hypoxic (0.5% O2, 5% CO2, 94.5% N2) conditions. The relative cell number was assessed 3 days later by methylene blue staining. Briefly, 100 μL methylene blue staining solution (0.5%/v/v in 50% ethanol and 50% double-distilled H2O) was added per well for 30 minutes followed by three washes in deionized water and air-drying. Methylene blue was eluted with 100 μL 0.1% n-laurosylsarcosine (1% w/v in PBS) for 30 minutes and absorbance was read at 620 nm on a Bio-Rad Laboratories (Mississauga, Ontario, Canada) Benchmark Plus microplate spectrophotometer. IC50 values were determined with PRISM version 4.00 software (GraphPad, San Diego, CA) using the sigmoidal dose-response (variable slope) function. The results are expressed as mean ± SD of at least three independent experiments per cell line done in triplicates or, in the case of PC-3 variants, as mean ± SD of three different variants per group.

Microvessel (CD31), apoptosis (terminal deoxynucleotidyl transferase–mediated dUTP nick end labeling), and proliferation (Ki-67) staining. For CD31-terminal deoxynucleotidyl transferase–mediated dUTP nick end labeling (TUNEL) containing, 6-μm cryosections were fixed with 4% paraformaldehyde, permeabilized with 0.1% Triton X-100 (v/v) and 0.1% sodium citrate (w/v), and incubated 20 minutes with 10% goat serum in PBS. Anti-CD31 antibody (1:200) was added in antibody diluting buffer for 1 hour at room temperature followed by Texas Red–conjugated Fab′ (goat anti-mouse IgG (7.5 μg/mL)) for 30 minutes. The TUNEL reaction was done according to the manufacturer’s instructions (In situ Cell Death Detection Kit, Roche Diagnostics, Indianapolis, IN), and nuclei were counterstained with DAPI (1 μg/mL in PBS for 2 minutes). Images were acquired as described above (fluorescence filters for TUNEL staining: 490 nm excitation/525 nm emission). Microvessel density and TUNEL labeling were assessed on two sections (>30 μm apart) per tumor (n = 3 tumors per group) and 5 to 10 randomly chosen fields (>400) per section. Single endothelial cells or clusters distinct from adjacent vascular structures were scored as microvessels.

For formalin-fixed tissues, 5 minutes of 350 W microwave irradiation in 0.1 mol/L citrate buffer (pH 6) preceded the TUNEL reaction. The signal was converted with peroxidase/diaminobenzidine substrate. Ki-67 staining was done as described recently (7). Next, the TUNEL- or Ki-67-positive cells per high-power field (>400) were counted (10 fields per tumor section, two sections per tumor, n ≥ 3 tumors per group).

Statistical analysis. All statistical analyses were done with PRISM version 4.00 software as indicated (GraphPad, San Diego, CA). The level of significance was set at P < 0.05.

Results

LDM cyclophosphamide exerts sustained antiangiogenic effects on PC-3 xenografts resulting in decreased intratumoral oxygen tension. LDM is thought to act mainly via antiangiogenic mechanisms (4). Consistent with this idea, the IC50 of 4-HC (preactivated cyclophosphamide) for inhibition of proliferation of PC-3 human prostate cancer cells in vitro using a 6-day metronomic protocol is >10-fold higher than for human umbilical vein endothelial cells (0.580 versus 0.045 μmol/L), revealing a selective “antiangiogenic window” as described for other cell lines and cytotoxic drugs (4). Established PC-3 xenografts treated with LDM cyclophosphamide given in the drinking water display a typical pattern of delayed-onset regression followed by a period of stable disease and finally eventual progression (31). To illustrate the in vitro antiangiogenic activity of LDM cyclophosphamide in this model, mice with established (∼300 mm3) PC-3 xenografts were started on LDM cyclophosphamide and the tumors were subsequently analyzed for microvessel counts after 1 week (at the start of tumor regression), 4 weeks (during the stable disease phase), and 7 weeks (during progression) of therapy. Microvessel density is independent of the tumor size over a range from 300 to 1,300 mm3 (∼P < 0.05, linear regression analysis) in both treated and untreated tumors. As shown in Fig. 1A, microvessel density is reduced by 42% after 1 week of therapy and, perhaps more importantly, remains suppressed during the tumor progression “breakout” phase. Concurrently, endothelial cell apoptosis is increased (Fig. 1B). Similar to the reduced microvessel density, a 43% decrease in Matrigel plug perfusion was seen with LDM cyclophosphamide treatment (Fig. 1C).

The antitumor effects observed with most antiangiogenic therapies are thought to be mediated primarily by increased hypoxia (or even anoxia; ref. 17). However, the relation between angiogenesis and tumor hypoxia is complex (34). In the absence of a “gold standard” for assessing tumor oxygenation (35), three different experimental approaches were applied. Two of these approaches involved the exogenous hypoxia marker EF5, a fluorinated nitroimidazole compound that binds to macromolecules under hypoxic conditions (36). The detection of EF5 adducts by the mouse monoclonal antibody ELK3-51 then allows for quantitative analysis of the degree of hypoxia by immunofluorescence or flow cytometry. Figure 2A shows representative tumor sections of control and LDM cyclophosphamide-treated xenografts of similar size (∼1,200 mm3). Image analysis revealed a significant increase in mean fluorescence, and hence hypoxia, already apparent after 1 week of treatment (Fig. 2A). At the end of the 7-week treatment period, mean fluorescence is increased even further. Similar results were obtained regarding median fluorescence (data not shown).
Flow cytometry of PC-3 xenograft single-cell suspensions revealed a significant decrease in the viable cell fraction of unsorted tumor cell populations after 7 weeks of LDM cyclophosphamide (Fig. 2C, 1). Interestingly, the hypoxic (EF5-positive) cell fractions were similar in end-stage control tumors (f1,200 mm3) and after 4 and 7 weeks of LDM cyclophosphamide, respectively, the latter being size matched with the control tumors (Fig. 2C, 2). However, histogram analysis showed a significant increase in mean and median fluorescence after 7 weeks of treatment (Fig. 2C, 3 and 4). The EF5 fluorescence intensity histograms of two representative tumors are depicted in Fig. 2D, showing increased ELK3-51-dependent fluorescence in the LDM cyclophosphamide-treated tumor compared with normal saline control.

With a separate set of tumors, the oxygenation status of PC-3 xenografts (mean tumor size, ∼1,500 mm3) under different therapy regimens was assessed using a polarographic oxygen electrode. As shown in Fig. 3, tumors subjected to LDM cyclophosphamide display increased hypoxia compared with normal saline control as indicated by a left shift in the histogram of the pooled data (P = 0.079, Mann-Whitney test) and an increase in the therapeutically important hypoxic fraction V5mm Hg from 59.8% to 71.3% (P < 0.001 for the cumulative fraction curves obtained by averaging individual data sets, normal saline versus LDM cyclophosphamide, Friedman test with Dunn’s multiple comparison).

The hypoxic cell cytotoxin tirapazamine exhibits antiangiogenic activity in vitro and in vivo. Various antivascular effects of...
Figure 2. LDM cyclophosphamide reduces oxygenation of PC-3 xenografts as assessed by EF5 immunofluorescence (A and B) and flow cytometry (C and D). A, relative EF5-related fluorescence intensity of frozen sections stained with ELK3-51 antibody increases steadily over the course of LDM cyclophosphamide treatment. Columns, mean; bars, SE. B, EF5 immunostaining (red) and nuclear counterstaining with DAPI (blue) of representative frozen sections (×200 magnification) of PC-3 xenografts (1,200 mm³). Bar, 100 μm. C, LDM cyclophosphamide treatment leads to a decreased fraction of viable tumor cells after 7 weeks (1). Despite a similar percentage of viable, hypoxic (EF5-positive) cells (2), the mean (3) and median (4) EF5-related fluorescence intensity increases significantly over a 7-week treatment course compared with normal saline control. The cell suspensions were prepared from tumors after administration of normal saline for 4 weeks (black columns; control tumors, 1,200 mm³) and LDM cyclophosphamide for 4 weeks (gray columns) and 7 weeks (white columns; size matched with control tumors). Columns, mean; bars, SE. D, representative flow cytometry histograms of a size-matched control tumor (mouse not injected with EF5) and tumors from the normal saline control (4 weeks) and LDM cyclophosphamide (7 weeks) groups, respectively. ns, \( P > 0.05; \) *, \( P < 0.05; **, \ P < 0.01; *** , \ P < 0.001 \) (one-way ANOVA, Newman-Keuls multiple comparison test).
tirapazamine have been postulated (24, 25, 27, 28). Accordingly, proliferation of bovine aortic endothelial cells is slightly but significantly more sensitive to tirapazamine under normoxic conditions and yet as sensitive under hypoxic conditions as various tumor cell lines tested (Fig. 4). Because the Matrigel plug perfusion assay mimics neoangiogenesis under hypoxic conditions (37), we assessed the effect of tirapazamine on plug perfusion. As shown in Fig. 1C, tirapazamine alone decreases perfusion more efficiently (75%) than LDM cyclophosphamide. Otherwise, for unknown reasons, the effect on microvessel density in end-stage tumors was less pronounced (Fig. 4B). The CD31-TUNEL costaining of end-stage tumors suggested additive antiangiogenic activity of the combination therapy (Fig. 4C; P = 0.056). However, neither the Matrigel plug perfusion assay nor the microvessel density analysis showed additive effects. Thus, although we have ostensibly confirmed the antiangiogenic activities of tirapazamine, such effects seem to be quite limited (at least in the PC-3 xenograft model).

The combination of LDM cyclophosphamide and tirapazamine delays the growth of PC-3 prostate, HT-29 colon, and MDA-MB-231 breast cancer xenografts, is well tolerated, and results in severely hypoxic tumors. Based on toxicity and antitumor efficacy, 20 mg/kg/d is the optimal metronomic dose of cyclophosphamide given in the drinking water, which can be applied over prolonged periods without major toxicities (7, 31, 38). In preliminary experiments, weekly i.p. administration of 25 mg/kg tirapazamine was found to be the regimen with the optimal therapeutic index when combined with LDM cyclophosphamide. In the PC-3 model (Fig. 5A), tirapazamine was used either upfront (i.e., from the first day of treatment of established xenografts) or delayed (i.e., after 4 weeks of treatment; corresponding to the end of the plateau phase). LDM cyclophosphamide monotherapy doubled the time to end point (TTE; defined as mean tumor size of 1,500 mm³) compared with saline control. Conversely, tirapazamine monotherapy had only very limited activity either given upfront or at relapse/progression. However, when combined with LDM cyclophosphamide, both upfront and delayed tirapazamine prolonged the TTE by 2.6- and 2.9-fold, respectively. This corresponds to higher than additive activity (additive activity calculated as \[ \frac{(TTE_{tirapazamine \text{ upfront}} / TTE_{\text{normal saline}}) \times (TTE_{\text{LDM cyclophosphamide}} / TTE_{\text{normal saline}})}{2.25\text{-fold}} \]), suggesting synergism. A beneficial effect of the combination regimen was also observed in s.c. implanted HT-29 human colon adenocarcinoma xenografts and the orthotopic MDA-MB-231 human breast cancer xenograft model (Fig. 5C).
Pilot experiments in C.B-17 SCID mice with combined LDM cyclophosphamide and tirapazamine showed an unexpected degree of toxicity (as measured by weight loss). This may be due to the DNA damage caused by both cyclophosphamide and tirapazamine coupled with the SCID phenotype that results from a mutation in the gene coding for the catalytic subunit of the DNA-dependent protein kinase, implicated in DNA damage repair, particularly the repair of DNA double-strand breaks (39). Indeed, primary skin fibroblasts derived form SCID mice were found to be significantly more sensitive to tirapazamine under normoxic conditions, and similarly sensitive under hypoxic conditions, compared with fibroblasts obtained from NIH Swiss nude mice (data not shown). By using NIH Swiss nude mice, the combination regimen was well tolerated for prolonged periods as shown for the PC-3 experiment (Fig. 5B, similar data obtained for HT-29 and MDA-MB-231 experiments not shown). The weight loss seen at the end of the experiment is potentially treatment related but might also reflect advanced disease, aging, and frequent occurrence of dental problems at this stage, the nature of which remains to be elucidated.

Complete tumor eradication was not achieved with the combination regimen. PC-3 variants obtained from end-stage tumors of various treatment groups displayed similar tirapazamine IC_{50} for proliferation inhibition under both hypoxic and normoxic conditions compared with control (i.e., variants obtained from animals injected normal saline; Fig. 6A). Colony formation assays of selected PC-3 variants also did not show significant differences (data not shown). Acquired tumor cell resistance to the cytotoxic activity of tirapazamine is therefore an unlikely explanation for the observed progression under treatment. Alternatively, improved oxygenation could theoretically explain reduced tirapazamine activity, although it seems that the opposite is actually the case. Indeed, polarographic oxygen electrode measurements show more severe hypoxia in end-stage PC-3 xenografts under the combination treatment compared with control tumors of similar size (Fig. 3A, similar data obtained for HT-29 and MDA-MB-231 experiments not shown). Despite severe hypoxia, these tumors show significantly increased proliferative activity as assessed by Ki-67 staining (Fig. 6B), which is mirrored by an increased apoptotic rate (Fig. 6C). In fact, the high proliferative activity might contribute to the severe hypoxia seen (40).

**Discussion**

The LDM treatment concept has shown some very promising results in advanced breast, prostate, and ovarian cancer as well as sarcomas and melanomas (4, 5, 41), albeit in several of mostly limited size phase II clinical trials. Moreover, it seems that the antitumor effects, at least in preclinical studies, can be potentiated by combinations of LDM with (a) targeted antiangiogenic drugs (6, 11, 42), (b) conventional cytotoxics, either as an upfront short course of MTD chemotherapy ("chemoswitching"; ref. 42) or as intermittent bolus dose injections of lower than MTD doses along with LDM ("fast" plus "slow" metronomic chemotherapy; ref. 43), or (c) targeted antitumor agents with accidental antiangiogenic activity, such as trastuzumab (44). Here, we present another refinement designed to improve efficacy and delay resistance (i.e., the combination of LDM cyclophosphamide with the hypoxic cell cytotoxin, tirapazamine). This regimen displays impressive antitumor activity that is accompanied by a favorable toxicity profile and moreover could potentially be applied clinically as a convenient oral regimen because an oral formulation of tirapazamine displays reasonable pharmacokinetics (45).
The rationale for the LDM cyclophosphamide and tirapazamine combination was 2-fold. As we have shown recently, LDM-based regimens might eventually select for hypoxia-resistant tumor cell populations (15) that are potentially targetable with tirapazamine; in addition, the tumor vasculature itself might be affected by tirapazamine in various ways (24, 25, 27, 28). Despite enhanced activity of combined LDM cyclophosphamide and tirapazamine, complete tumor eradication was not achieved. As such, our study confirms that the benefits of LDM, and antiangiogenic therapies in general, are limited by acquired resistance. Regarding the latter, our study has broader implications. Tumor relapses under antiangiogenic therapies interfering with the VEGF pathway could result from “rebound angiogenesis” supported by alternative angiogenic factors and/or vascular remodeling leading to a more resistant vascular phenotype (13, 14). Conversely, we show here that LDM cyclophosphamide results in decreased microvessel density that is sustained even during tumor progression in the absence of remodeling. In addition, the relapsing tumor phenotype is characterized by severe hypoxia and high tumor cell proliferative activity. The proven antiangiogenic activity of the LDM cyclophosphamide schedule applied and the documented sustained reduction in microvessel density suggest an antiangiogenic basis of the hypoxia observed. Alternative explanations, such as reduced

3 U. Emmenegger and A. Kouri, unpublished observations.
Figure 6. Inhibition of proliferation by tirapazamine of PC-3 variants grown under normoxic and hypoxic conditions (A) and proliferation (Ki-67; B) and apoptosis (TUNEL; C) staining of PC-3 xenografts.  

A, effect of tirapazamine on proliferation of PC-3 variants derived from tumors of various treatment groups was assessed under normoxic (20% O₂; gray columns) and hypoxic (0.5% O₂; white columns) conditions and is expressed as IC₅₀.  

Columns, mean of three different variants from the same treatment group; bars, SD. There is no indication for acquired resistance to tirapazamine.  

B, quantification of Ki-67-positive cells per ×400 field reveals very active tumors in terms of proliferation under LDM cyclophosphamide with or without tirapazamine therapy. Representative sections (×200) from tumors treated with normal saline, tirapazamine, LDM cyclophosphamide, and combination (all treatments given upfront).  

Columns, mean; bars, SE. C, TUNEL labeling of the same tumors as in (B) mirrors the proliferative activity.  

Apoptosis index: number of labeled cells per ×400 field. Representative sections (×400). CNS, normal saline control; ECT, LDM cyclophosphamide + tirapazamine upfront; LCT, LDM cyclophosphamide + tirapazamine delayed; LT, normal saline followed by tirapazamine at progression. ns, P > 0.05; *, P < 0.05; **, P < 0.01; ***, P < 0.001 (one-way ANOVA, Newman-Keuls multiple comparison test).
oxygen transport capacity (43) and increased oxygen consumption by tumor cells surviving single, MTD-like cyclophosphamide doses (46, 47), are unlikely in the LDM context. However, we cannot exclude that the high proliferative activity seen in the relapsing tumors contributes to the decreased oxygenation. To sum up, our observations are in line with the notion that normalization takes place in the PC-3 model under LDM cyclophosphamide treatment, although we did not perform a detailed analysis of the vessel structure.

It remains to be seen whether the changes found in the PC-3 model using LDM cyclophosphamide can be generalized. Selectedο2 measurements confirm the suppressed oxygenation of HT-29 and MDA-MB-231 xenografts that progressed under LDM cyclophosphamide (data not shown). Stollting et al. showed similar trends (reduced microvessel density and increased proliferation) in human non–small cell lung cancer xenografts under metronomic trofosfamide, an alkylating agent from the oxazaphosphorine family, like cyclophosphamide (48). In contrast to our results, LDM topotecan monotherapy results in a certain degree of vascular remodeling in Wilm's tumor xenografts (49). Possibly, the hypoxia-inducible factor-1α (and hence indirectly VEGF) inhibitory effects of the topoisomerase I inhibitor topotecan (50) may be the basis for the remodeling seen. The complex and multifactorial antiangiogenic effects of LDM (4) suggest that such regimens are potentially less prone to simple escape mechanisms, such as switching to other angiogenic factors, as documented for specific VEGF pathway inhibitors (13).

The antitumor effects of tirapazamine monotherapy were only minor in all three models tested, consistent with the findings of others (51). Moreover, although we found a slight but not significant Ω2 decrease in tirapazamine-treated PC-3 xenografts, Masunaga et al. described an increase in Ω2, which they attributed to selective killing of hypoxic tumor cells (52). The reduced oxygenation in our model might be partially a direct consequence of the antivascular effects of tirapazamine (24, 25, 27, 28). In fact, it was recently speculated that the antitumor effects of tirapazamine are more dependent on antivascular rather than hypoxic cytotoxic effects (25). In this regard, the antivascular activities of tirapazamine might eventually compromise its own delivery. Furthermore, reduced tumor oxygenation might hinder the already poor penetration characteristics of tirapazamine by increased metabolic consump-

tion (53). Such an effect would be expected to be more pronounced in the LDM cyclophosphamide plus tirapazamine combination regimen. Consequently, the most hypoxic tumor parts potentially would not be affected by tirapazamine, which in turn could result over time in a tumor composition characterized by a net increase of more severely hypoxic cells. Insufficient tirapazamine pharmacokinetic characteristics might therefore be an explanation for the tumor progression seen in vivo in absence of in vitro (pharmacodynamic) resistance of the various PC-3 variants tested. It remains to be seen whether newer generations of hypoxic cytotoxins will be able to overcome limitations of tirapazamine (54).

Whereas excessive hypoxia therefore might be detrimental in vivo, the cytotoxic activities of tirapazamine in vitro are linearly dependent over a wide range (55). Accordingly, Blumenthal et al. showed that hypoxic cell cytotoxin therapy can be optimized in vivo as a function of tumor oxygenation (56). In addition, stratification for the degree of hypoxia before start of treatment significantly improved the results of a clinical trial involving chemoradiation with or without tirapazamine (57). Because the percentage of human tumors being considered "hypoxic" varies depending on the tumor entity (57, 58), it is conceivable that tumors considered not sufficiently hypoxic for successful tirapazamine administration could be made so by using LDM protocols.

In conclusion, besides combinations of LDM regimens with targeted or accidental antiangiogenic drugs, combined LDM cyclophosphamide and weekly tirapazamine represents a novel way to improve the antitumor effects of metronomic chemotherapy protocols. Both cyclophosphamide and tirapazamine can be given orally. Hence, in terms of clinical translation, such a regimen would combine convenience with a favorable toxicity profile. Newer generations of hypoxic cytotoxins might be even more beneficial by overcoming pharmacokinetic shortcomings of tirapazamine. It remains to be seen whether criteria can be defined that allow selecting for patients that benefit most from such an approach. Finally, our results may clearly have relevance to other antiangiogenic drugs or therapies, including targeted agents that induce significant hypoxia after prolonged treatment (13).

References
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