Repeated tumor oximetry to identify therapeutic window during metronomic cyclophosphamide treatment of 9L gliomas

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Abstract. Malignant gliomas are aggressive and angiogenic tumors with high VEGF content. Consequently, approaches such as metronomic chemotherapy, which have an anti-angiogenic effect, are being investigated. However, a lack of an appropriate technique that can facilitate the identification of vascular changes during antiangiogenic treatments has restricted therapeutic optimization. We have investigated the potential of tumor pO_2 as a marker to detect vascular changes during metronomic chemotherapy. Electron paramagnetic resonance (EPR) oximetry was used to repeatedly assess tumor pO_2 during metronomic cyclophosphamide treatment of subcutaneous 9L tumors. The 9L tumors were hypoxic with a pO_2 of 5.6-8 mmHg and a tumor volume of 247-300 mm^3 prior to any treatment. Tumor pO_2 increased significantly to 19.7 mmHg on day 10 and remained at an elevated level until day 33 during 4 weekly treatments with 140 mg/kg cyclophosphamide. A significant decrease in the tumor volume on days 21-31 occurred in the cyclophosphamide group, while the tumor volume of the control group significantly increased during measurements for two weeks. A significant tumor growth delay was achieved with two weekly treatments of cyclophosphamide plus radiotherapy (4 Gy x 5) as compared to control, cyclophosphamide and radiotherapy alone groups. The results indicate the potential of EPR oximetry to assess tumor pO_2 during metronomic chemotherapy. The ability to identify the duration of an increase in tumor pO_2, therapeutic window, non-invasively by EPR oximetry could have a significant impact on the optimization of antiangiogenic approaches for the treatment of gliomas. This vital information could also be used to schedule radiotherapy to enhance therapeutic outcome.

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Introduction

Gliomas are highly angiogenic tumors with rapid infiltrative growth and profound microvascular proliferation (1). Despite improvements in surgery and radiotherapy; the prognosis of glioma patients has remained poor (2,3). Consequently, new methods are urgently needed to improve and synergize strategies for the treatment of gliomas. The presence of tumor hypoxia (pO_2 <10-15 mmHg) further compromises the outcome by stimulating glioma progression, aggressive phenotypes, metastases and resistance to therapies (4-6). Tumor hypoxia cannot be predicted by tumor type or size and therefore must be measured. The significant influence of hypoxia on tumor progression and resistance to therapies makes it a critical factor that could be targeted to achieve therapeutic benefit for patients with malignant gliomas. Additionally, the dynamics of tumor pO_2 during the course of treatment is characteristic of the tumor microenvironment and proliferation, therefore, pO_2 can potentially be used to monitor treatment efficacy and identify responders and non-responders.

Several methods have been developed to modulate tumor hypoxia, but these were largely unsuccessful in enhancing therapy, possibly because techniques were not available to assess the changes in pO_2 that affected the outcome of the therapy. With the development of *in vivo* EPR oximetry, it is now possible to obtain repeated measurements of tumor pO_2 as often as desired (7-10). EPR oximetry will be especially useful to monitor the effect of antiangiogenic approaches, such as metronomic chemotherapy, which involves treatment with anticancer drugs in 1/3 to 1/10 of the maximum tolerated dose on a frequent schedule (daily, weekly or bi-weekly) (11-13). Metronomic regimens also have shown benefits in controlling cancers that have developed resistance to given chemotherapeutics (14). The primary mechanism is the inhibition of tumor angiogenesis by directly killing endothelial cells in the tumor neovasculature, rapidly proliferating tumor cells, and suppressing the mobilization of bone marrow derived circulating endothelial prognitor cells (13-15). The effect on tumor endothelial cells is expected to result in a transient normalization of tumor vasculature, which in turn could prevent tumors from progressing to a proangiogenic state that may lead to invasiveness and an increased likelihood of metastases

(16). The vascular normalization should also enhance drug delivery and oxygen (pO_2) into the tumors, thereby providing a therapeutic window, which if exploited, could improve the outcome of chemoradiation.

However, the effect of metronomic chemotherapy is likely to vary with the dose and the interval between doses and therefore may significantly affect therapeutic outcome. A lack of method to repeatedly measure and follow vascular normalization and/or increase in tumor oxygenation has been a rate limiting step in rationally translating the metronomic approach into the clinic and in developing protocols for combination therapies.

We hypothesize that a repeated measurement of tumor pO_2 by EPR oximetry will provide crucial information that will indicate vascular normalization during metronomic chemotherapy. The temporal changes in the tumor pO_2 could be exploited to optimize existing metronomic protocols and synergize it with radiotherapy. We report the effect of metronomic cyclophosphamide treatments on the subcutaneous 9L tumor pO_2 and growth. The temporal changes in tumor pO_2 were followed and the tumors were irradiated at the time of an increase in pO_2 during metronomic chemotherapy to assess therapeutic outcome.

Materials and methods

Animal and tumor model. All procedures were in strict accordance with the NIH Guide for the Care and Use of Laboratory Animals and were approved by the Institutional Animal Care and Use Committee of Dartmouth Medical School. 9L is a chemically induced rat brain tumor that has been used for more than 30 years in studies related to growth and response to therapies (17). The 9L cells were grown *in vitro* in DMEM medium with 10% FBS and 1% penicillin-streptomycin. For injection, the cells were trypsinized and suspended in DMEM, without serum or additives. The tumors of 6-8 mm in diameter were obtained in 14-16 days by subcutaneous injection of 100 μl cell suspension ($4x10^5$ cells) with a 25-gauge needle in the left flank of anesthetized (2% isoflurane with 30% O_2) SCID mice (Charles River Laboratories, MA).

Implantation of oximetry probe (LiPc) for pO_2 measurements using EPR oximetry. EPR oximetry at two sites in each tumor was used for tumor pO_2 measurements. Oxygen-sensitive lithium phthalocyanine (LiPc) crystals were synthesized in our laboratory and were used as an oximetry probe in this study. The EPR spectra of the LiPc crystals exhibit a single sharp EPR line with a line width highly sensitive to pO_2 changes and reflect the average pO_2 on the surface of the crystals. The high density of the unpaired spins along with the narrow intrinsic line width of LiPc crystals allow the measurements of pO_2 in the tumor using a few crystals with a total diameter of ~200 μm (7,9,18). Once the tumors reached a size of 6-8 mm in diameter, the mice were anesthetized (2% isoflurane with 30% O_2), and two aggregates of LiPc crystals (40-50 μg each) were injected into the tumors 4 mm apart and at a depth of 2 mm from the tumor surface using 25-gauge needles. EPR oximetry using these LiPc deposits provided simultaneous pO_2 measurements at two sites of each tumor (7-9,18).

For tumor pO_2 measurements, the mice were anesthetized (1.5% isoflurane, 30% O_2) and positioned between the magnet poles of 1.2 GHz L-band EPR spectrometer equipped with a microwave bridge and an external loop resonator specially designed for *in vivo* experiments (18,19). The two LiPc implants in each tumor were located along the lateral axis. The animals were positioned in the spectrometer such that this axis was parallel to the direction of the applied gradient in the main magnetic field. A gradient of up to 3.0 G/cm was used to separate the EPR signals of the two LiPc implants in each tumor. The external loop resonator was placed close to the surface of the tumor and pre-treatment (baseline) EPR measurements were acquired for 30 min on day 0. The EPR spectra were averaged for 1 min each to improve the signal to noise ratio. Typical settings for the spectrometer were incident microwave power, 4-6 mW; magnetic field center, 400 gauss; scan range, 2 gauss; modulation frequency, 27 kHz. Modulation amplitude was set at less than 1/3 of the EPR line width. During EPR measurements, the temperature of the animals was monitored using a rectal probe and maintained at 37±0.5˚C by keeping the body warm with a thermostatically controlled heated pad and a flow of warm air. The inspired gas of 30% O_2 (balanced N_2) provides blood pO_2 values in the normal range, and is routinely used in our laboratory. The EPR line widths were converted to pO_2 using a calibration determined for the batch of LiPc crystals used in this study. The tumor pO_2 and volume measurements were continued for 4-6 weeks.

Metronomic cyclophosphamide (CPA). The antiangiogenic and direct cytotoxic effect of metronomic CPA provide a combination approach that is absent with most angiogenesis inhibitors. The maximum tolerated dose of CPA in SCID mice is reported to be 265 mg/kg body weight (20). The choice of 140 mg/kg CPA for metronomic dosing in this study is based on the earlier finding of an antiangiogenic effect by weekly administration of 170 mg/kg CPA (21). Additionally, 140 mg/kg CPA on weekly schedule has been used to investigate the mechanistic pathways of its antiangiogenic effect and the possibility of its combination with other approaches (22,23).

CPA (Sigma-Aldrich Co., MO) was prepared in phosphate-buffered saline (PBS, Mediatech Inc., VA) immediately before each treatment and filtered through a 0.22-μm sterile Millex filter units (Millipore Corp., MA). CPA was administered to the mice by i.p. injections at 140 mg/kg body weight.

Irradiation. The 9L tumors were irradiated by 4 Gy for 5 consecutive days using a Varian Linear Accelerator (6 MeV electron beam, 6 cm x 6 cm applicator). The mice were anesthetized (1.5% isoflurane, 30% O_2) and positioned on the irradiator bed. The beam was collimated with a lead shield to irradiate the tumor while sparing the surrounding normal tissue. This machine is maintained by the Radiation Oncology Section at Dartmouth-Hitchcock Medical Center (DHMC) and is available through the Norris Cotton Cancer Center Shared Service.

Tumor volume measurements. The tumor length and width were measured by vernier caliper (Mitutoyo Corp., Japan) before tumor pO_2 measurements on each day. The tumor volumes were calculated using the formula: $\pi/6$ x length x $width^2$. This is a

well established procedure for tumor volume measurement of peripheral tumors and has been used routinely in our experiments (9,24).

Immunoblot. Vascular endothelial growth factor receptor 2 (VEGFR-2) and rabbit IgG antibodies were purchased from Cell Signaling Technology (Beverly, MA, USA). 9L tumors of the control and CPA treated groups were collected on days 0, 7 and 14 and then immediately pulverized in liquid nitrogen using a mortar and pestle until a fine powder was obtained. After washing with ice cold PBS, the pellets were lysed on ice for 30 min in RIPA buffer (2 ml/1 mg tissue weight) (Pierce, Rockford, IL, USA) and centrifuged for 10 min at 2000 x g to pellet particulate matter. This process was repeated twice and the supernatants combined for protein concentration determination using the Nanodrop A280 assay. Samples were diluted to a concentration of 3 μg/μl in SDS loading buffer and boiled for 5 min. Protein (60 μg) was separated using 10% SDS-PAGE gels and then transferred onto nitrocellulose membranes for immunodetection as suggested by the manufacturer. The immunoreactive bands were visualized with ECL (Amersham Biosciences, NJ, USA) and the exposure time was adjusted to keep the integrated optical densities within a linear and non-saturated range. Band signal intensity was quantified by densitometry using a flatbed scanner (Epson Perfection 3200 Photo) and imaging software (AlphaEaseFC). Molecular weight markers (Cell Signaling) were used as molecular mass standards. A one-way ANOVA on ranks was performed for overall comparisons with the Student-Newman-Keuls post-hoc test used to determine differences between groups. The level of significance accepted *a priori* was $p<0.05$.

Experiment design. The mice were randomly assigned to following groups: i) CPA (140 mg/kg x 4), n=7; ii) control (vehicle only), n=7; iii) CPA (140 mg/kg x 2), n=6; iv) CPA (140 mg/kg x 2) + 4 Gy x 5 (day 10-day 14), n=6; v) CPA (140 mg/kg x 2) + 4 Gy x 5 (day 5-day 9), n=6; and vi) 4 Gy x 5 (day 5-day 9), n=8. The changes in the tumor pO_2 observed in group i) and iii) were used to design the timing of radiation for groups iv) and v). In group iv), the tumors were irradiated when an increase in pO_2 was observed (day 10-day 14), while in group v) the tumors were irradiated when they were hypoxic (day 5-day 9) during CPA treatments. In group vi), the mice were treated with vehicle only and the tumors irradiated on day 5-day 9. Groups i), iii), iv), v) and vi) are represented as 4CPA, 2CPA, 2CPA/Irrd/Oxic, 2CPA/Irrd/Hypoxic and Irrd alone, respectively, in rest of the manuscript. First a pre-treatment (baseline) tumor pO_2 and volume were measured (day 0) in all the groups and then the measurements were continued for 2-6 weeks. The control group was terminated on day 14 due to very large tumor masses as per the IACUC guidelines at Dartmouth.

Data analysis. The pO_2 measurements obtained from the two LiPc implants of each tumor were pooled to determine average tumor pO_2. A paired t-test was used to determine the statistical significance of the changes in pO_2 within the group, and an unpaired t-test was used to determine the statistical significance between groups at the same time-points. The tests were two-sided, and a change with a p-value <0.05 was considered statistically significant. The data are expressed as the mean ± SEM; n is the number of animals in each group (only + SEM bars are shown in the figures for the sake of visual clarity).

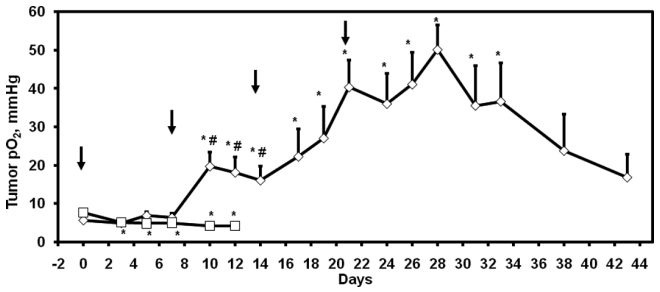

Figure 1. Temporal changes in 9L tumor pO_2 in i) 4CPA (◇), n=7 and ii) control (□), n=7 groups over days. The mice were treated with CPA (140 mg/kg, i.p) on days 0, 7, 14 and 21 (indicated by arrows) in the 4CPA group, and equal volumes of PBS in the control group. Mean + SEM, n is the number of mice in each group. *p<0.05 compared with the baseline (pre-treatment); #p<0.05 compared with the control group.

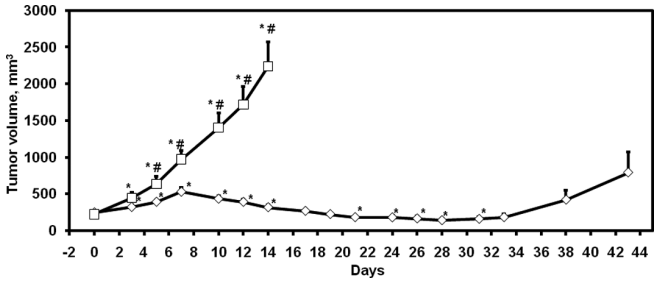

Figure 2. The 9L tumor volume in i) 4CPA (◇), n=7 and ii) control (□), n=7 groups over days. The mice were treated with CPA (140 mg/kg, i.p) on days 0, 7, 14 and 21 in 4CPA group, and equal volumes of PBS in the control group. Mean + SEM, n is the number of mice in each group. *p<0.05 compared with the baseline; #p<0.05 compared with the CPA group.

Results

Effect of weekly CPA (140 mg/kg x 4) treatment on 9L tumor pO_2. The baseline tumor pO_2 of the 4CPA and the control groups were 5.6±1 mmHg and 7.6±1.1 mmHg, respectively and these were not significantly different, Fig. 1. A significant increase in the tumor pO_2 of the 4CPA group from day 10 (19.7±3.7 mmHg) as compared with the baseline and the control group was observed. The tumor pO_2 remained significantly elevated till day 33 and then gradually declined to the baseline. On the other hand, a significant decrease in the tumor pO_2 of the control group from day 3, as compared to the baseline, was observed.

Effect of weekly CPA (140 mg/kg x 4) treatment on 9L tumor growth. The pre-treatment tumor volumes of 4CPA and the control were 248±25 and 225±26 mm^3 and were not significantly different, Fig. 2. The tumor volume of 4CPA group increased significantly from day 3 till day 14 as compared to the baseline and then significantly decreased between days 21-31. Thereafter, a gradual increase in the tumor volume

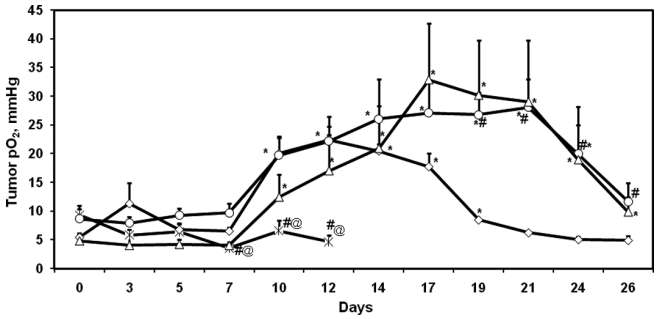

Figure 3. Temporal changes in 9L tumor pO_2 in i) 2CPA (◇, 140 mg/kg x 2), n=6; ii) 2CPA/Irrd/Oxic (○, 140 mg/kg x 2 + 4 Gy x 5, day 10 to 14), n=6; iii) 2CPA/Irrd/Hypoxic (△, 140 mg/kg x 2 + 4 Gy x 5, day 5 to 9), n=6 and iv) Irrd alone (∗, 4 Gy x 5, day 5 to 9), n=8 groups over days. In 2CPA/Irrd/Oxic and 2CPA/Irrd/Hypoxic groups, the tumors with similar tumor volume were irradiated when they were oxygenated or hypoxic respectively during the treatments with CPA. Mean + SEM, $^{*}p<0.05$ compared with baseline; $^{\#}p<0.05$ compared with 2CPA; $^{@}p<0.05$ compared with 2CPA/Irrd/Oxic groups.

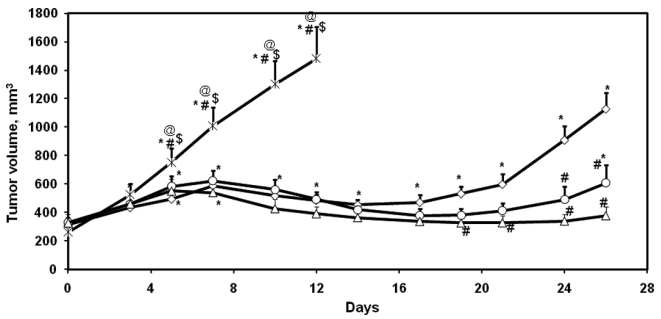

Figure 4. The 9L tumor volume in i) 2CPA (◇, 140 mg/kg x 2), n=6; ii) 2CPA/Irrd/Oxic (○, 140 mg/kg x 2 + 4 Gy x 5, day 10 to 14), n=6; iii) 2CPA/Irrd/Hypoxic (△, 140 m/kg x 2 + 4 Gy x 5, day 5 to 9), n=6; iv) Irrd alone (∗, 4 Gy x 5), n=8 groups over days. Mean + SEM, $^{*}p<0.05$ compared with baseline; $^{\#}p<0.05$ compared with 2CPA; $^{@}p<0.05$ compared with 2CPA/Irrd/Oxic; $^{\$}p<0.05$ compared with 2CPA/Irrd/Hypoxic groups.

was observed but these changes were not significantly different from the baseline. In the control group, a significant increase in the tumor volume from day 3 as compared to baseline was evident. The tumor volumes of the control group were also significantly greater than the 4CPA from day 5 to 14.

Effect of CPA (140 mg/kg x 2) treatment and radiotherapy (4 Gy x 5) on 9L tumor pO_2. The changes in the 9L tumor pO_2 of 2CPA, 2CPA/Irrd/Oxic, 2CPA/Irrd/Hypoxic and Irrd alone groups are summarized in Fig. 3. No significant differences in the baseline tumor pO_2 were observed between groups and the control. Additionally, no significant change in the tumor pO_2 of the Irrd alone group as compared to the baseline or the control was noted. A significant increase in the tumor pO_2 of 2CPA group from day 10 to 19 was observed, which then returned to the baseline. The temporal changes in the tumor pO_2 of 2CPA group were similar to the 4CPA group. A significant increase in the tumor pO_2 of 2CPA/Irrd/Oxic group occurred from day 10 and the pO_2 remained elevated until day 24 as compared to the baseline. The tumor pO_2 of 2CPA and 2CPA/Irrd/Oxic groups were also significantly

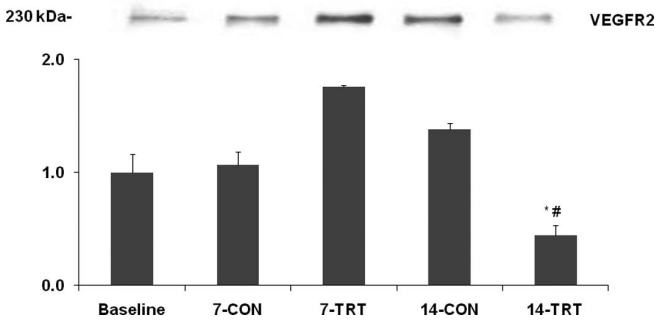

Figure 5. Western blot analysis of VEGFR2 in the 9L tumors. Fold change in the protein content of the 2CPA treated (7-TRT and 14-TRT) and control (7-CON and 14-CON) groups on day 7 and day 14 compared to the baseline (day 0). Mean + SEM, $^{*}p<0.05$ compared with 7-TRT; $^{\#}p<0.05$ compared with 14-CON groups.

higher than the controls from day 10 and day 5, respectively. A significant increase in the tumor pO_2 from day 7 to 14 was observed in 2CPA and 2CPA/Irrd/Oxic groups as compared to Irrd alone. Additionally, the tumor pO_2 of 2CPA/Irrd/Oxic group was significantly higher than 2CPA from day 19 to 24. These results were used to design 2CPA/Irrd/Hypoxic group, in which the tumors with similar tumor volumes but hypoxic were irradiated with 4 Gy x 5 on day 5 to 9.

Effect of CPA (140 mg/kg x 2) treatment and radiotherapy (4 Gy x 5) on 9L tumor growth. All the groups had similar baseline tumor volumes on day 0, Fig. 4. The tumor volume of the 2CPA group was significantly higher from day 5 as compared to the baseline. A significant increase in the tumor volume of the 2CPA/Irrd/Oxic group was also observed from day 5 to 12 and day 24 to 26 as compared to the baseline.

The tumors irradiated with 4 Gy x 5 alone had a significant increase in the tumor volume from day 3 to 14 as compared to the baseline but these changes were not significantly different from the control group. A significant tumor growth delay in the 2CPA/Irrd/Oxic and 2CPA/Irrd/Hypoxic group was observed as compared to 2CPA alone group on day 24 to 26 and day 21 to 24, respectively. Whereas, the tumor volume of the 2CPA, 2CPA/Irrd/Oxic and 2CPA/Irrd/Hypoxic groups were significantly smaller than the control and Irrd alone from day 7. However, no significant difference in the tumor growth between 2CPA/Irrd/Oxic and 2CPA/Irrd/Hypoxic group was observed.

Changes in tumor VEGFR2 during weekly 140 mg/kg CPA. VEGFR2 is a well known receptor tyrosine kinase which promotes angiogenesis by endothelial cell differentiation, proliferation, chemotaxis and new vessel formation (25). Immunoblotting analysis indicated no significant change in the VEGFR2 content on days 7 and 14 in the control group compared to the baseline (day 0), Fig. 5. Also no significant change in VEGFR2 was observed between the control and treated groups on day 7. However, a significant decrease in the VEGFR2 on day 14 compared to day 7 in the 2CPA group was observed. These changes in VEGFR2 were also significantly lower than the controls.

Discussion

Antiangiogenic approaches, such as by metronomic chemotherapy, may hold promise in the treatment of highly angiogenic gliomas. The ability to follow the effect of metronomic chemotherapy using markers that could be assessed repeatedly during therapy could be used to optimize metronomic schedules and guide the combination with other therapeutic modalities, such as radiotherapy.

We have systematically investigated the effect of weekly CPA treatments on the subcutaneous 9L tumor pO_2 and growth over days. We sought to further take advantage of an increase in tumor pO_2 to establish an optimized schedule for radiation therapy in the context of weekly antiangiogenic CPA treatments. The 9L tumors were hypoxic and a further decrease in the tumor pO_2 with increase in tumor volume was evident in the control group. No significant effect of 4 Gy x 5 on 9L tumor growth indicates the radioresistant nature of these tumors. Treatment with four weekly doses of CPA led to a significant increase in the tumor pO_2 which remained at an elevated level throughout the treatment. A similar increase in the tumor pO_2 was also observed with two weekly treatments of CPA. These treatments also resulted in a relatively slow increase in the tumor volume as compared to the control for up to two weeks followed by significant tumor shrinkage in the subsequent weeks.

The increase in the tumor pO_2 during metronomic CPA is likely due to vessel normalization resulting in an increase in blood flow to the tumor. A unique feature of metronomic cyclophosphamide schedule is that it has both a direct cytotoxic and antiangiogenic effect unlike most angiogenic inhibitors (15,26). Both, the weekly CPA schedule (21) and continuous CPA in the drinking water were shown to induce antiangiogenic activity (11,15) and are thought to generate severe hypoxia or anoxia in tumors (27). Unlike the continuous CPA treatment such as through the drinking water which maintained hypoxia (28); we have earlier observed an increase in tumor oxygenation and drug uptake with two weekly CPA thus suggesting a functional improvement of tumor vasculature (10).

A significant decrease in the VEGFR2 content in the 2CPA group occurred on day 14. These results are in agreement with earlier reports of an increase in tumor pO_2 which coincided with an increase in the antiangiogenic markers (29,30). The changes in the tumor pO_2 were used to schedule radiotherapy when the tumors were well oxygenated or hypoxic to determine the therapeutic outcome. The irradiation of the tumors at the time of an increase in tumor pO_2 led to a significant delay in the tumor growth as compared to the CPA and irradiation alone groups. Under most antiangiogenic treatments, the repetitive or continuous administration of angiogenic inhibitors prevents endothelial cells from recovering, thus leading to capillary 'drop out' and tumor collapse. In the case of weekly CPA schedule, such as ours, the increased CPA uptake and oxygenation has increased the radiotherapeutic sensitivity of tumor cells as well as endothelial cells leading to dramatic tumor shrinkage. The mechanism of similar effects on the tumor growth in 2CPA/Irrd/Oxic and 2CPA/Irrd/Hypoxic groups could be due to several factors such as inhibition of angiogenesis, inhibition of cell proliferation and stimulation of apoptosis. It is likely that the combination of such effects masked the effect of tumor hypoxia on radiotherapeutic response; this warrants further investigation.

A significant increase in the tumor pO_2 was also evident in mice treated with 2CPA/Irrd/Oxic and 2CPA/Irrd/Hypoxic groups as compared to 2CPA alone. Several possible mechanism have been suggested for oxygenation post-irradiation such as reduced oxygen consumption by radiation-damaged cells, cell loss leading to tumor shrinkage and improved microcirculation (31). Nevertheless, these results confirm that the antiangiogenic effect of metronomic CPA will enhance tumor pO_2, thereby will provide a therapeutic window that could be exploited to merge it with other therapeutic approaches. However, it should be emphasized that the antiangiogenic effect is likely to vary with the drug used, dose, interval between doses, and also with tumor types. Using polarographic electrodes or hypoxic markers, a decrease, no change, and also an increase in tumor pO_2 has been reported using various angiogenic inhibitors in different tumor models (32).

In conclusion, EPR oximetry provides temporal changes in the tumor pO_2 during metronomic CPA treatment of 9L tumors. Results indicate a significant increase in tumor pO_2 i.e., a therapeutic window, during weekly CPA treatments. A significant delay in the tumor growth was achieved when metronomic chemotherapy was combined with fractionated radiotherapy. With non-invasive repeated tumor oximetry by EPR, it is now possible to systematically investigate the effect of antiangiogenic approaches on tumor pO_2 in pre-clinical models and develop protocols that could potentially enhance therapeutic efficacy in a clinical setting. EPR oximetry is currently being tested for repeated tumor pO_2 measurements in patients with peripheral tumors undergoing chemo and/or radiotherapy (18,33). We anticipate that EPR oximetry will be a valuable tool to study the effect of various treatments on tumor oxygenation and use this vital information to optimize such treatments and guide its efficacious combination with other therapeutic approaches.

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