Photodynamic Therapy with Verteporfin in the Radiation-induced Fibrosarcoma-1 Tumor Causes Enhanced Radiation Sensitivity


ABSTRACT

Photodynamic therapy (PDT) with verteporfin (lipid form of benzo- porphyrin derivative, benzo[4,5]benzoporphyrin derivative monocyclic A) was used to treat radiation-induced fibrosarcoma tumors before X-ray treatment. When verteporfin was injected 3 h before light irradiation, the tumor partial pressure of oxygen (pO₂) rose from a pretreatment value of 2.8 ± 1.6 to 15.2 ± 6.9 mm Hg immediately after light application was complete (P = 0.048). When the optical irradiation was given 15 min after verteporfin injection, the pO₂ decreased slightly after treatment [i.e., 6.8 ± 1.6 mm Hg (pretreatment) versus 4.1 ± 0.3 mm Hg (posttreatment)], whereas control tumor pO₂ did not change significantly. In vitro study of the cellular oxygen consumption rate before and after PDT treatment indicated the consumption rate decreased linearly with delivered optical dose and quantitatively matched the loss of cell viability as measured by a mitochondrial tetrazolium assay. Doppler measurements show that red cell flux is still patent immediately after treatment, indicating that oxygen should still be delivered to the tumor. Computational simulations of the oxygen supply from the vessels and the consumption from mitochondrial activity confirmed that if oxygen consumption is decreased in the presence of unhindered blood flow, the tumor oxygenation should rise, and the hypoxic fraction of the tumor should decrease. Combination treatments with PDT delivered (100 J/cm²) optical dose, with 1 mg/kg benzo[4,5]benzoporphyrin derivative monocyclic A injected 3 h before treatment) after radiation treatment (10 Gy from 300 keV source) were compared with PDT delivered simultaneously with radiation. Tumor regrowth assay showed that the delays to reach double the tumor volume for PDT alone and radiation alone were 2.7 ± 1.6 and 3.2 ± 1.7 days, respectively. When radiation was given before PDT, the delay was 5.4 ± 1.4 days, and when PDT was given at the same time as radiation, the delay was 8.1 ± 1.5 days. This observation indicates that the combined effect in the latter case was greater than additive (P = 0.049).

INTRODUCTION

PDT is a treatment modality for dysplastic tissues, which uses a light-activated drug to kill targeted regions of tissue (1–4). A photosensitizer with delivery vehicle is applied to the tissue topically or systemically, and moderate intensity light is used to selectively excite these molecules to their first excited triplet state, which is then efficiently quenched by molecular oxygen to produce singlet state oxygen. The cellular response to high doses of singlet oxygen is complex but generally causes phospholipid peroxidation leading to cellular membrane damage and vessel occlusion-mediated ischemia, ultimately causing necrosis or rapid apoptosis in the parenchyma. The mechanism for cellular killing is thought to be different from that of radiation treatment, where unrepaired DNA strand breaks provide the destructive effect. Several studies have investigated the combination effect of PDT and γ-radiation, and many have concluded that the combined treatment provides an additive, but not synergistic, effect (5, 6). In this study, we examine a non-vascular-targeting type of PDT treatment with verteporfin photosensitizer (7), which induces increased oxygen tension in the tumor (8) and, when combined with radiation treatment, induces a greater than additive effect in tumor killing.

Previous studies have examined specific interactions between PDT and radiation therapy to determine what the effect of combined therapy would be. Several early studies have examined whether the photosensitizer molecule itself acts as a radiosensitizer, and whereas some have shown effects with hematoporphyrin derivative and ionizing radiation (9), the majority of studies have concluded that there was no radiation sensitivity enhancement due to the photosensitizer (10–12). In comparison, many more studies have examined the utility of combined PDT treatment with radiation treatment, but most have been carried out in monolayer cell culture (5, 13–15), largely with the conclusion that synergistic effects are not observed. However, studies with both aluminum phthalocyanine and aminolevulinic acid have indicated that timing may be a key factor in determining whether synergistic effects are observed (13, 16) because synergistic effects are observed at longer temporal separations between the radiation and PDT treatments. In addition, any tissue response characteristics that occur would not be present with in vitro assays. In vivo studies have demonstrated mixed effects: Wilter et al. (17) demonstrated no synergistic effect of PDT and radiation in tumors; and Benstead and Moore (18) indicated that combined treatment of meta-tetra(hydroxyphenyl) chlorin (mTHPC-PDT) with X-rays increased the normal tissue damage but that the effect of time sequence of the two treatments upon outcome was not significant. To date, none of these studies have examined specifically whether PDT could be delivered in a manner that changes the tissue metabolism such that, when used in conjunction with radiation therapy, it provides a superadditive or synergistic effect.

Our studies have shown that mitochondria function can be acutely impaired when verteporfin treatment is applied directly to the cells and that this effect is observed in vivo as well, as measured by a loss of NADH fluorescence (19). This effect is likely a direct result of the fact that BPD-MA (the photoactive molecule in verteporfin) predominantly localizes on the mitochondria membranes, and therefore PDT action would likely cause widespread mitochondria damage (20, 21). If PDT can be used to halt cellular metabolism shortly after treatment, then there should be a net beneficial effect by increasing the oxygen available to redistribute to previously hypoxic areas (8). The hypothesis of this work was that if a PDT treatment could be applied that preserved blood flow immediately after treatment yet reduced the oxygen consumption by the parenchyma cells, then the tumor tissue oxygenation would increase. This reoxygenation phenomenon would
be most dominantly observed where preexisting hypoxia is present due to the larger diffusion distances from capillaries (22). In our studies, we have chosen to follow this effect in the RIF-1 tumor model (23), which is known to have large regions of hypoxic cells (22, 24, 25) and has also been well studied in our previous studies (22, 26–29) as well as in PDT studies of several other groups (25, 30–35).

pO2 changes that occur in vivo during PDT are complex and have been shown to vary with many factors (36). The necessity for oxygen to be present for tissue killing by PDT has been shown both in vitro and in vivo (31), and the consumption of oxygen in this process can be readily observed as an acute decrease of tissue pO2 (37–39). These observations led to a suggestion that high optical dose rates could lead to less tumor killing due to the transient depletion of oxygen (39). This concept has been supported by the observation that the effectiveness of treatment decreases in some tumor models when high optical dose rates are used for treatment (35, 40–42). In addition to this transient decrease in tumor oxygenation, it has been observed that rapid and permanent reduction in blood flow and tumor oxygenation (43–45) can occur due to massive vascular occlusion during or soon after PDT when a large fraction of the exogenous photosensitizer is present in the vasculature (46). Whereas this vascular-targeting approach to delivering PDT has been a dominant line of study, it is also interesting to consider the longer time regime, where the photosensitizer has largely leaked out of the vasculature or been cleared from the blood.

Linuma et al. (46) have shown that verteporfin is initially confined to the vasculature at 5 min postinjection but that at 1 h postinjection, it appears to be predominantly perivascular in the orthotopic rat prostate NBT-II tumor. Fingar et al. (45) showed that the effect of vascular stasis appears acutely in chondrosarcoma and muscle when light is delivered 15 min after injection with verteporfin-based therapy but that it was inhibited or delayed by several hours when light was given 3 h after injection. Furthermore, Major et al. (47) have shown that blood vessels can dilate after verteporfin treatment, potentially increasing the flow after verteporfin-based PDT. Our own studies have indicated that even when verteporfin-based PDT is given in a vascular-targeting approach, the response is heterogeneous at the microscopic scale, indicating that individual vessels vary in their response to the treatment. Our studies in the RIF-1 tumor model with verteporfin treatment have shown that blood flow is still patent immediately after treatment and decreases steadily during the hours following therapy but that if an incubation time of 3 h is used, then the decrease in blood flow is only to 55% of the pretreatment value. Thus, current evidence would indicate that when verteporfin-based PDT with optical irradiation is given several hours after injection, the blood vessel occlusion does not predominantly occur immediately during treatment, preserving blood flow during and after therapy.

In contrast to blood flow, the metabolic consumption rate of the tumor parenchyma cells has not been adequately examined in PDT. Whereas this parameter is very challenging to measure directly in vivo (48–51), it could be significantly altered by PDT for photosensitizers that primarily target the mitochondria. The rate of oxygen consumption would be inhibited and the mechanism would depend upon the light fluence, irradiance, and localization of the photosensitizer. It has been observed that one of the earliest events in PDT damage in vitro is the loss of mitochondrial membrane integrity, which would lead to a loss of cellular respiration and, ultimately, apoptosis (52–56). In necrotic cell death, loss of respiration would be instantaneous, but it is also likely that sublethal damage to the mitochondria induces a transient or permanent reduction in cellular respiration. The continuation of blood flow coupled with acute reduction in cellular respiration could increase or redistribute oxygen during PDT. This effect, combined with reduced killing in regions that were hypoxic before PDT, could lead to reoxygenation of chronically hypoxic areas, as has been observed in our earlier study with both verteporfin and amino levulinic acid-protoporphyrin IX treatment in the RIF-1 tumor (8).

In this study, changes in tumor pO2 were quantified both during and after treatment with non-vascular-targeting verteporfin-PDT. The cellular oxygen consumption rate was determined for RIF-1 cells in vitro. Computer simulations are used to interpret the observations and examine what the change in hypoxic fraction is expected to be. This treatment is then combined with X-ray irradiation to look for beneficial combinatorial effects of the dual therapy. Preliminary evidence suggests that non-vascular-targeting PDT could be a good mechanism to decrease the hypoxic fraction of tumors, thereby increasing the efficacy of radiation therapy.

MATERIALS AND METHODS

Cells and Tumor Model. RIF-1 cells were used for in vitro and in vivo studies. RIF-1 cells were originally supplied by James B. Mitchell (National Cancer Institute), and they were grown in vitro in RPMI 1640 supplemented with 10% fetal bovine serum, glutamine, and antibiotics and grown with no more than four passages from the original stock. In vitro studies were carried out with the cells in 96-well plates, given 3 days between plating and use for treatment. For in vivo studies, cells were grown in large culture flasks, removed, resuspended in medium without fetal bovine serum, and injected at 4 × 105 cells/mouse in 50 μl volume. Intradermal injection on the upper right leg was used in female C3H/HeJ (5–6 weeks old) mice (The Jackson Laboratory, Bar Harbor, ME), approximately 11–14 days before the anticipated treatment time.

Photosensitizer. The photosensitizer, verteporfin, was obtained from QLT Phototherapeutics Inc. (Vancouver, British Columbia, Canada) for this experimental study. Verteporfin is the active ingredient in Verteporfin For Injection (VFI), the lipid formulated drug that is marketed as Visudyne for the treatment of ocular disease. The photoactive molecule present in this preparation is BPD-MA (57). For in vivo use, the drug as supplied was reconstituted in PBS at 0.2 mg BPD-MA/ml and then injected into the mice through the lateral tail vein to give a dose of 1 mg BPD-MA/kg body weight. For in vitro use, BPD-MA was reconstituted in PBS at 1 mg BPD-MA/ml solution in PBS and diluted in medium to expose cells to 1 μg/ml BPD-MA. For both in vivo and in vitro work, an incubation period of 3 h was used in these experiments, except where otherwise specified. The drug was kept in the dark and frozen between experiments.

Light Delivery. A diode laser system up to 200 mW average power was used throughout these studies (Applied Optronics, South Plainfield, CT), with a wavelength of 690 nm. The beam was delivered to the animals through a 140 μm fiberoptic and expanded onto the tumor or cell plate in a circular top-hat beam, using a fiberoptic collimator (Thor Labs, North Newton, NJ). The beam diameter for in vivo work was 1.1 cm, using an irradiance of 133 mW/cm2, and for in vitro work, it was a 2-cm-diameter beam for an irradiance of 63.7 mW/cm2. In vivo light treatment was given transectaneously, with the animals cleanly shaven before treatment.

In Vitro PDT Treatment. Cells were plated in black plastic 96-well plates with a transparent bottom (Fisher Scientific, Springfield, NJ) at a density of 5000 cells/200 μl medium/well. The cells were used for PDT treatment after 3 days of growth in 95% oxygen with 5% CO2 at 37°C in a humidified incubator. On the day of treatment, the medium was replaced with 100 μl/well medium with 1 μg/ml BPD-MA in the verteporfin preparation. After 3 h in the incubator, the BPD-MA was decanted off, cells were rinsed once with HBSS, and then 100 μl of medium were added to each well. Cells were illuminated in groups of four wells, with blank wells between the treated groups to ensure that each group of wells received the correct dose. For each 96-well plate, squares of four were treated with increasing total light dose with 8 groups/plate, including the control with no light. Cells were then assayed for cell viability or cellular oxygen consumption.


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Cell Viability Assay (MTS). Cell viability was assayed 24 h after treatment, using the MTS assay kit (CellTiter 96 AQueous; Promega, Madison, WI), which is used to measure the reduction of a tetrazolium compound by the cellular mitochondria, producing an optically active soluble formazan. After PDT treatment, the medium from the 96-well plate was removed, and the MTS tetrazolium reagent was added. After a 1-h incubation at 37°C, the absorbance of each well was measured in an automated plate reader (Thermo Max; Molecular Devices, Menlo Park, CA) at the 490 nm absorption of the MTS soluble formazan product. For data analysis, the absorbance values from groups of wells that received the same light dose were averaged, and the data were normalized by the average value from the control (no light) wells.

Cellular pO₂ Measurement. Immediately after PDT, cells were trypanosed and suspended in medium, and cell viability was determined by trypan blue exclusion assay before use in the oxygen consumption measurement. Cells were resuspended at 2 × 10⁶ viable cells/ml in complete RPMI 1640. Just before use, the cell suspension was diluted to 10⁴ cells/ml with a solution of 10% dextran in complete medium and used for oxygen consumption assay.

The rate of oxygen consumption was measured from the cells using EPR. Cells suspensions with dextran were extracted in 0.100 ml volumes and mixed with a neutral nitroxide, 1⁵⁵ Pd N-D (4-oxo-2,6,6,6-tetramethylpiperidine-1-yl-⁵⁵ N-1-oxyl) at 0.2 mm (Cambridge Isotope Laboratories, Quebec, Canada) in a 4-μl aliquot. This mixture was then drawn into glass capillary tubes, which were then sealed with Critoseal (Sherwood Medical, St. Louis, MO). The sealed tubes were placed into quartz ESR tubes, and samples were maintained at 37°C by a heated flow of gas through the resonator. All spectra were recorded on a Bruker EMMX EPR spectrometer operating at 9 GHz. Because the resulting linewidth was proportional to oxygen concentration, oxygen consumption rates were obtained by measuring the concentration repeatedly over 10–20 min, and the slope of the resulting data was determined by linear regression. Three repeated trials were completed for each sample of cells, and the slopes were averaged.

Tumor pO₂ during In Vivo PDT. Animals were anesthetized with inhalation of isoflurane at 1.5% mixed with 26% oxygen in a continuous flow, delivered by a nose cone to loosely cover the head. The animal was maintained at 37°C on a heated water pad, and warm air flowed over the animal. The animals were used for continuous monitoring of tumor pO₂ before, during, and after the treatment using EPR oximetry as described below. In the first treatment group, tumors were treated with light 3 h after the verteporfin injection. In the second group, the photosensitizer was injected just 15 min before optical irradiation, and these animals were followed for tumor pO₂ before, during, and after treatment. This type of short-term treatment is thought to elicit a strong vascular occlusion response and thus provides a contrast to the effect from a cellular targeting treatment, where a 3-h delay is given between injection and irradiation. The third group was control animals that were treated with light alone to simulate the effects of light absorption in the tissue without photosensitizer.

Tumor pO₂ was monitored with an oxygen-sensitive EPR probe material, synthetic LiPc, which can be implanted into the tissue and provide stable measurements of tissue pO₂ over several weeks (27, 58–61). Initial experiments demonstrated that pO₂ could be monitored with EPR during the PDT treatment without any interaction between the probe and the photosensitizer or light (8, 29). Small particles (approximately between 50 and 200 μm) of LiPc were implanted in the animal tumors at least 24 h before PDT treatment to allow for resolution of any acute effects due to the injection. A 23-gauge syringe needle was used to implant the autoclaved, dry material at a depth of 1–3 mm within the tumor. Only one implantation of oxygen-sensitive material was necessary, and pO₂ could be monitored as often as desired during the experiment.

The animals were placed in an L-band (1.2 GHz) EPR spectrometer with a microwave bridge custom-built for measurements of tissue pO₂ in small animals. The external loop resonator was positioned over the tumor to record the EPR signal of the LiPc within the tissue. Typical settings for the spectrometer were incident microwave power of 50 mW, magnetic field of 400 G, scan range of 0.5 G, modulation frequency of 27 kHz, modulation amplitude of 15 mG, and scan time of 30 s. The settings were not significantly changed between animals in this study nor during an acquisition of an individual animal. After accumulation of the linewidths as a function of time before, during, and after treatment, the data were fit with a custom-written software program to match the Voigt line shape of the EPR spectrum and extract the linewidth. The LiPc was calibrated to determine the ambient pO₂ from the linewidth, such that the data could be used to calculate effective tissue pO₂ during the experiment.

Laser Doppler Flow Measurement. Laser Doppler measurements of RBC flux were taken on a section of animals given PDT treatment, using an implantable fiberoptic probes (Moor Instruments, Wilmington, DE). The measurement of red cell flux can be used to monitor microcirculatory function and provides a proportionate measurement of blood flow, assuming that hematocrit levels are constant throughout a given measurement. Two fiber probes had external diameters of 200 μm and were implanted in the tumor tissue through holes made with a 26-gauge needle immediately before insertion. Both probes were implanted within the light field, and automated data collection was done with the supplied software on a computer. Blood flow was sampled at 40 Hz, and data points were averaged together over 5-min and 30-min periods after the experiment. All flow values during the treatment time were standardized to the average value during the first 2 min after probe implantation. Animals were divided into three control groups: (a) no light, no drug; (b) drug, no light; and (c) light, no drug. Two treatment groups were used including a 15-min as well as one treatment group using a 3-h interval between verteporfin injection and light irradiation. Control animals were further divided into three subgroups: (a) no verteporfin, no light; (b) verteporfin, no light; and (c) light, no verteporfin. Verteporfin was injected i.v. at 1 mg/kg BPD-MA concentration 3 h before animal treatment. In the treatment group, blood flow was monitored for 2 min, followed by 12 min of light treatment, followed by 1 h of blood flow measurement. Blood flow during laser treatment was meaningless because during this time, the irradiation light saturated the Doppler probe. All values were normalized to their pretreatment value before averaging together the numbers from each animal in each treatment group. The animals in the PDT treatment group (as opposed to controls) were not sacrificed after the 30-min time point but were later reanesthetized, and their tumors were reimplemented with blood flow probes to measure flow at 6 h posttreatment.

Combined PDT and Radiation Therapy Studies. Experimental studies examining the combination effect of PDT and radiation therapy were completed in this tumor model. The injection of BPD-MA or saline (for control) was given 3 h before treatment in all cases. Light at 690 nm wavelength was delivered to the PDT-treated animals while the animal was positioned in the irradiator cone. An optical irradiance of 133 mW/cm² was used over 12 min, for a total optical dose of 100 J/cm². Radiation was a single dose of 10 Gy (300 keV, 10 mA, half value layer (HVL) = 2.33 Gy/min). The treatment groups were as follows: (a) sham-irradiated controls; (b) radiation treatment alone; (c) PDT alone; (d) radiation treatment followed by PDT; and (e) PDT with radiation treatment given simultaneously. In the fourth treatment group, X-ray radiation was given for the full 3-min treatment time after the photosensitizer was injected 3 h after photosensitive injection, and then optical irradiation was given 30 min after the X-ray radiation. In the fifth treatment group, the optical irradiation was given 3 h after the drug injection, and during the last 3 min of the optical irradiation, X-ray irradiation was also given to the tumor at the same time. This latter timing would result in the tumor being exposed to X-rays during the maximal reoxygenation time of the tumor tissue.

Irradiation of the 10-Gy dose using approximately 300 keV was delivered in 3 min from a Pantak Therapax 300 Orthovoltage irradiator, using a 2-cm collimation cone. This cone was placed in intimate contact with the mouse leg, surrounding the tumor. The end of the collimation cone was transparent plastic, allowing radiation and optical treatment to occur at the same time, as required in the last group, where PDT and radiation were simultaneously applied. All animals were treated with the cone in place, even those treated with sham irradiation and PDT alone, to ensure that equal doses of light, including changes in the beam from reflection and refraction through the irradiator cone, were given to all groups.

Tumor Regrowth Assay. Mice were assigned to the different treatment groups in the combined radiation and PDT study in a manner that produced matching initial tumor volumes at the time of treatment to minimize any systematic errors associated with groups having different initial volumes at the time of treatment. The resulting treatment effect was assayed by calculation of the tumor volume, as determined by measurement of the three major axis dimensions (volume = length × width × height/2). The time for a tumor to reach double its volume on the day of treatment was calculated for each animal separately by estimating the data by a linear fit to the logarithmic growth curve versus time and estimating when it had reached twice the tumor volume as on
the day of treatment. The average values from these times to double in volume were calculated for each treatment group.

To analyze the tumor regrowth data and obtain estimates of the mean doubling time and its SE, individual growth curve data were combined for each group and fitted using a mixed effects model (28, 62). It was assumed that after day 3, the tumor regrows exponentially, which corresponds to a linear function when plotted on a semi-log graph. To address mice heterogeneity, a mixed effects modeling technique was applied, which has been previously applied in O’Hara et al. (28) and described by Demidenko (62). The time for a tumor to reach double its volume on the day of treatment was calculated for each group along with the respective SE using the delta-method (63). These SEs give rise to the group comparison using Z-test to test for significant differences between the mean values of the difference treatment groups.

**Oxygen Distribution Simulation Studies in RIF-1 Tumors.** A previously reported simulation study was used to evaluate the effect of changes in metabolic consumption on the resulting tumor tissue pO2 (22). This study used a finite element solution to the steady-state diffusion equation to solve for the oxygen concentration everywhere within an arbitrary volume of tissue. The differential diffusion equation is given in steady state by

$$D \nabla^2 C_{o2}(r) - k_{met}(r, O_2) + S_{o2}(r) = 0$$

(1)

where $C_{o2}(r)$ is the oxygen concentration at position $r$. $D$ is the diffusion coefficient for oxygen in tissue (which is generally taken to be spatially independent), $k_{met}(r, O_2)$ is the metabolic oxygen consumption rate, and $S_{o2}(r)$ is the supply of oxygen by the capillaries at each point $r$.

The capillary geometries for the simulation were derived from eight separate H&E-stained sections of RIF-1 tumor tissue, digitized, and manually thresholded. The capillary oxygen supply rates were estimated based on fitting to boundary information, which was given by pimonidazole staining of adjacent sections of the tissue. This staining provides demarcation of regions of hypoxia, thereby allowing estimation of the neighboring capillary oxygen supply rates, assuming that the oxygen diffusion coefficient is known ($D = 2 \times 10^{-5}$ cm/s). Our earlier study indicated that a metabolic oxygen consumption rate of $k_{met} = 10 \mu$mol/s was appropriate to simulate an oxygen consumption rate in the untreated cells was 1.84 ± 0.14 mmol/min/million cells. When treated with light alone, there are small increases in this oxygen consumption rate on the order of 0.2 mmol/min/10^6 cells, and when treated with the photosensitizer and light, there is a monotonic decrease in oxygen consumption correlated to the delivered light dose. At the highest light dose used here (16 J/cm^2), the oxygen consumption rate fell to 0.37 ± 0.04 mmol/min/10^6 cells. The data for these measurements are plotted in Fig. 2, shown by open symbols (squares for treated cells and circles for control), with the absolute units of oxygen consumption plotted on the right-hand vertical axis. The control group received light doses without incubation with photosensitizer.

The cell viability as measured by MTS assay is plotted on the same graphs. The tumor oxygen distribution was studied in RIF-1 tumors in mice treated with verteporfin-based PDT and control animals with light alone but no photosensitizer ($n = 5$). There were two treatment groups, each of which received 1 mg/kg BPD-MA in the verteporfin preparation ($n = 5$ each) either 15 min or 3 h before light treatment. In both cases, the light was delivered with a total dose of 144 J/cm^2 at 690 nm wavelength. The error bars in the figure represent the SD, and the values for the 3-h-treated group immediately after treatment (labeled after Tx on the graph) are significantly different from control values ($P < 0.048$).

**RESULTS**

**In Vitro Tumor pO2.** Measurements of tumor pO2 were taken in control and treated tumor-bearing animals. Mean ± SD tumor volume for all groups at the time of treatment was 203 ± 23 mm^3 (range, 110–300 mm^3). There was no significant difference between the tumor volumes of the groups at the time of treatment. The EPR probe was placed within the top 2–3 mm of tumor tissue to ensure that it was reporting from a region that had a full effect of the PDT. This measurement is localized and was repeated at several different times before, during, and after the treatment in each animal. Typically, the pO2 was recorded for 10 min before light irradiation, then recorded every minute throughout the treatment, and recorded for 10–15 min after the treatment. The mice were then reanesthetized at 1, 4, and 24 h posttreatment for pO2 measurement, requiring approximately 10–15 min. The average values at each time point were calculated separately for each animal for pretreatment, during treatment, immediately after treatment, and 1, 4, and 24 h posttreatment. The pO2 values for each group of animals were averaged together, and the SDs were calculated. These values are shown in Fig. 1.

Control animals were given the laser light but no photosensitizer injection. The control animals all had pO2 values initially at 3.6 ± 1.1 mm Hg, indicating that the tumors have a high hypoxic fraction, as has been shown in previous studies (22, 61). Throughout the laser treatment and afterward, the control animals maintained hypoxic pO2 values averaging 3.7 ± 1.2 mm Hg. In the treatment group, the initial pO2 was 2.8 ± 1.0 mm Hg. Initial changes in pO2 had a high variance between animals, with some increasing in pO2 immediately, some staying constant, and some fluctuating. By the end of the treatment period, all five of the treated tumors had risen significantly in their pO2 (8). The final value immediately after treatment was 15.2 ± 6.9 mm Hg, which is significantly different from the control value, as calculated by a paired Student’s t test with $P = 0.048$. At 1 h posttreatment, the pO2 was back to within control or pretreatment values. A group was also included with the photosensitizer injected just 15 min before treatment, which allows observation of the “vascular-targeting” type of therapy. In this case, the average pO2 decreased slightly during treatment while staying in the hypoxic range (i.e., 6.8 ± 1.6 mm Hg before light treatment and 4.1 ± 0.3 mm Hg immediately after treatment).

**In Vitro Cellular Oxygen Consumption and Viability.** Measurements of oxygen in suspensions of RIF-1 cells show that the oxygen consumption rate of untreated cells is higher than that of cells treated with BPD-MA-based PDT, as shown in Fig. 2. The average oxygen consumption rate in the untreated cells was 1.84 ± 0.14 mmol/min/million cells. When treated with light alone, there are small increases in this oxygen consumption rate on the order of 0.2 mmol/min/10^6 cells, and when treated with the photosensitizer and light, there is a monotonic decrease in oxygen consumption correlated to the delivered light dose. At the highest light dose used here (16 J/cm^2), the oxygen consumption rate fell to 0.37 ± 0.04 mmol/min/10^6 cells. The data for these measurements are plotted in Fig. 2, shown by open symbols (squares for treated cells and circles for control), with the absolute units of oxygen consumption plotted on the right-hand vertical axis. The control group received light doses without incubation with photosensitizer.
shown both immediately after light treatment as well as 30 min and 6 h posttreatment. The PDT-treated animals are shown summarized in Fig. 3. The flux immediately after treatment is equivalent to the flux in the control groups of no drug and no light, as well as drug only. Interestingly, the light alone control group shows a modest increase in blood cell flux immediately after the light delivery. The treatment group then has a reduction in blood cell flux over a longer time scale, with a reduction to 70% at 30 min after PDT and to 55% at 6 h after PDT.

**Calculation of Changes in pO2 Based on Changes in Metabolic Consumption Rate.** Using the capillary distributions and values obtained from RIF-1 tumor sections in our previous study (22), oxygen histograms were simulated for the two conditions of \( k_{\text{met}} = 10 \, \mu M/s \) and \( k_{\text{met}} = 0 \, \mu M/s \). The values of \( pO_2 \) were summed from the images used, and the data are presented in a histogram format. These are shown in Fig. 4, a and b, respectively. The median \( pO_2 \) values for these two distributions are 2 and 7 mm Hg, respectively, indicating that when the metabolic oxygen consumption rate of the cells is minimal, the \( pO_2 \) of the tumor tissue could increase by a median of 5 mm Hg. These data predict that the hypoxic fraction (the fraction of the tumor less than 10 mm Hg \( pO_2 \)) is initially 98%, whereas after the change in metabolic consumption, the hypoxic fraction is reduced to 65%. This simulation represents the extreme case of zero oxygen consumption after treatment, which is not the case from the measurements. Nonetheless, the increase in oxygenation is monotonically correlated with the oxygen consumption rate of the cellular regions, and so the two extremes simulated here present a simulated maximal range for the effect observed.

**PDT Combined with Radiation Therapy.** Measurements of tumor volumes after treatment with radiation, PDT, or both are shown in Fig. 5. In this experiment, the five treatment groups were as follows: (a) control with saline injection alone; (b) radiation therapy alone with BPD-MA injection but no light; (c) PDT alone with sham X-ray irradiation; (d) X-ray irradiation followed by PDT; and (e) PDT and radiation treatment given together. The data in this graph show that the tumor regrowth rate in all groups is slightly lower than that of the control group but that these slopes are similar between all treated groups. The slopes of the natural logarithm of tumor volume versus days were 0.20, 0.11, 0.13, 0.13, and 0.11, respectively, for the five treatment groups. PDT alone (group 3) and radiation alone (group 2) induce the same approximate time for the tumor to reach twice the volume of the treatment size (i.e., doubling times were 8.3 and 8.9 days, from Table 1). In comparison, groups 4 and 5 indicate...
focuses on the effect of PDT in cellular respiration and pO₂ changes that occur in this parenchyma-targeting regime. The photosensitizer has been most used clinically for vascular targeting in the effect on the vasculature. It is important to note that whereas this provides preferential targeting to the parenchyma while minimizing intravascular concentrations have a metabolic lifetime of about 6 h on time scales that are longer than a few hours, whereas shown that the verteporfin photosensitizer leaks into the surrounding tissue after treatment, as well as increased tumor pO₂ (8). This work was carried out, and the histogram calculations shown in Fig. 4 represented the average values of each group, and the error bars are the SE. The tumor volumes on the day of treatment and for doubling the treatment volume are denoted by horizontal dotted lines.

increasingly better treatment effects, with doubling times of 11.0 and 13.7 days, respectively.

**DISCUSSION**

In this study, verteporfin-based PDT has been explored as an adjuvant to radiation therapy, using a 3-h interval between injection and irradiation because this time point allows continuous blood flow in tissue after treatment, as well as increased tumor pO₂ (8). This work began under the hypothesis that non-vascular-targeting of PDT could be achieved using a drug interval of several hours between injection of the photosensitizer and the time of optical irradiation. Studies have shown that the verteporfin photosensitizer leaks into the surrounding parenchyma on time scales that are longer than a few hours, whereas intravascular concentrations have a metabolic lifetime of about 6 h (46). Thus, a treatment protocol that allows several hours of latency provides preferential targeting to the parenchyma while minimizing the effect on the vasculature. It is important to note that whereas this photosensitizer has been most used clinically for vascular targeting in age-related macular degeneration treatment (64), it is not limited to this type of therapy in principle. A longer drug-light interval is key to minimizing the vascular occlusion within the tumor and maximizing the direct cell damage, which causes acute mitochondrial damage. Our blood flow studies with verteporfin treatment in the RIF-1 tumor show results similar to the observations of Fingar et al. (45) in the chondrosarcoma tumor. When the 3-h drug-light interval is used, then the flow decreases after treatment, but at a slower rate than when 15 min is used. When the 15-min drug-light interval is used, the flow decreases steadily, decaying to 50% of initial values at 1 h after treatment and down to zero at 6 h after treatment. The potential applications for vascular-targeting versus parenchyma-targeting therapies are quite different, and yet little study has been devoted to contrasting these two potential dosimetry regimes (3, 21, 65–67). This study focuses on the effect of PDT in cellular respiration and pO₂ changes that occur in this parenchyma-targeting regime.

**Mitochondrial Function Is Inhibited after Verteporfin PDT.** In our experiments, cells that have been treated in vitro show a reduction in oxygen consumption rate (shown in Fig. 2). These data indicate that the change in oxygen consumption is directly proportional to the loss of cell mitochondrial function, as measured by the MTS assay. It is not surprising that these two assays should be proportional because they essentially both measure mitochondria activity, but because the MTS assay has been shown to be proportional to long-term cell survival in other studies, it is likely that the loss of oxygen consumption rate indicates acute damage to the mitochondria that can ultimately cause cell death. This change in oxygen consumption is also directly correlated to the optical dose delivered, so that the effect is likely due to singlet oxygen-mediated cellular death. The fact that these changes are observed immediately after the light treatment is an indicator that the predominant effect causing cell death is necrosis or some acute damage mechanism.

**Tumor pO₂ Increases Immediately after PDT.** In vivo tumor pO₂ measurements indicated that the tissue oxygen increased acutely in response to the treatment (Fig. 1). Our studies show that blood flow is not significantly reduced immediately after this type of treatment in the RIF tumor. Whereas this flow eventually degraded over time for all treatment groups, this indicates that there is a time period after PDT in this treatment regime where oxygen is still being delivered to the tissue. Because we observe that oxygen consumption is acutely reduced by the PDT treatment, we conclude that the tissue pO₂ could rise in response to therapy in which the blood vessels are not acutely occluded. This observation also agrees with the results we have reported for treatment of the RIF-1 tumor with aminolevulinic acid, where the rise in tumor oxygenation was also observed (8), and the decay in the oxygen level after treatment was observed to decay over the time course of about 1 h, indicating that this time period was probably when blood was flowing. Further study of the micro-regional kinetics after PDT would be useful to help characterize this phenomenon.

Simulations of oxygen distribution within the RIF-1 tumor tissue were carried out, and the histogram calculations shown in Fig. 4 present representative values that indicate the relative effect that changes in cellular oxygen consumption rate would have. In Fig. 4a, the histogram is very similar to that typically observed in the RIF-1 tumor model, on average, showing a dominant hypoxic fraction and a large fraction less than 5 mm Hg (22). By changing the oxygen consumption rate in these simulations to zero, the oxygen distribution in the tumor is simply given by the distribution of capillary pO₂ values that supply the region. In this case, the median pO₂ significantly increases from 2 to 7 mm Hg. This model approach assumes that the blood flow is not altered at all during therapy, in agreement with our measurements here. These simulations illustrate that changes in metabolic consumption rate can have a measurable effect on the tumor pO₂ value, decreasing the hypoxic fraction from 98% initially to 65% after loss of oxygen consumption. Alterations in blood flow would change the supply rate of oxygen and further perturb the measured pO₂, yet our studies did not demonstrate significant changes in flow. The limitation of this calculation is that it is for the specific tumors studied in our earlier paper (22) and that because this tumor is highly heterogeneous, it is likely that the effect will be higher in some regions and lower in other regions.

These observations have implications for PDT, suggesting that PDT-induced cellular damage may be used to increase oxygen in tumor tissue, including regions that were in a chronically hypoxic state. In our case, the pO₂ of the RIF-1 tumor increased from an

![Tumor volume vs. days after treatment](image)

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**Fig. 5. Average tumor volume is plotted relative to the number of days after treatment (days posttreatment, X axis) for the five treatment groups [control (n = 6 mice), radiation only (n = 5), PDT alone (n = 6), radiation followed by PDT (n = 7), and PDT together with radiation (n = 7)]. The points represent the average values of each group, and the error bars are the SE. The tumor volumes on the day of treatment and for doubling the treatment volume are denoted by horizontal dotted lines.**

---

**Table 1 Tumor doubling time and regrowth delay**

<table>
<thead>
<tr>
<th>Group</th>
<th>Treatment</th>
<th>Time for tumor to double initial volume</th>
<th>Regrowth delay in doubling time</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Control</td>
<td>5.6 ± 0.4</td>
<td>2.7 ± 1.6</td>
</tr>
<tr>
<td>2</td>
<td>Radiation only</td>
<td>8.3 ± 1.7</td>
<td>3.2 ± 1.7</td>
</tr>
<tr>
<td>3</td>
<td>PDT only</td>
<td>8.9 ± 1.7</td>
<td>3.2 ± 1.7</td>
</tr>
<tr>
<td>4</td>
<td>Radiation then PDT</td>
<td>11.0 ± 1.5</td>
<td>5.4 ± 1.4</td>
</tr>
<tr>
<td>5</td>
<td>PDT and Radiation</td>
<td>13.7 ± 1.6</td>
<td>8.1 ± 1.5</td>
</tr>
</tbody>
</table>
average of 3.6 mm Hg (control) to 15.6 mm Hg immediately after treatment. This would be sufficient to raise the average tumor oxygenation out of the region that is considered radiobiologically hypoxic and allow it to be effectively targeted by conventional radiation therapy. Whereas microscopic regions of hypoxia could still exist, the overall average oxygenation is sufficiently high that the beneficial effect of the oxygen enhancement ratio should be apparent. Thus, PDT-induced tumor reoxygenation could be used as a pretreatment to radiation therapy, thereby enhancing the radiation killing effect. These effects are quite time dependent, and therefore measurements of the tissue PO2 could be a useful guide for timing subsequent therapy with ionizing radiation. Ultimately, the tumor oxygenation falls back to hypoxic levels, indicative of something else happening at longer times, and this would correlate with the reduction in blood flow observed in our study at longer times after irradiation. Additional studies are needed to explore this effect and the potential for synergistic interaction with radiation therapy.

**Combined PDT and Radiation Therapy Induce a Greater than Additive Killing Effect.** After the observation of increased tumor PO2 in response to PDT, it was concluded that there would likely be a significant benefit of PDT and radiation therapy if used together in this model. The tumor regrowth delay data shown in Fig. 5 and analyzed in Tables 1 and 2 support this hypothesis. Whereas the tumor doubling times increased from 5.6 ± 0.4 days in the control group to 8.3 ± 1.7 and 8.9 ± 1.7 days, respectively, for the radiation and PDT alone groups, the combined treatment times illustrated that the order of the radiation versus PDT delivery matters significantly. A doubling time of 11.0 ± 1.5 days was observed for the delivery of radiation first, followed by PDT, and an increase to 13.7 ± 1.6 days was observed when PDT was used as the pretreatment adjuvant for the radiation. Using the tumor regrowth delay (last column of Table 1) as the measure of damage, this indicates that group 4 treatment (when radiation is given before PDT) induces an additive effect of the measure of damage, this indicates that group 4 treatment (when radiation. Using the tumor regrowth delay (last column of Table 1) as observed when PDT was used as the pretreatment adjuvant for the 2.7 days) and PDT alone (3.2 days) where given separately, because 2.7 ± 3.2 = 5.9 ± 1.9 days. This additive delay is in good agreement with the observed effect of group 4 treatment at 5.4 ± 1.4 days. In the last group (group 5), when PDT and radiation were delivered together, the regrowth delay was 8.1 ± 1.5 days, indicating an effect that is more than additive (68, 69). Student’s t test comparisons were completed between groups 2 and 3 and between group 5 and all others to determine whether there were significantly differences (P < 0.05), with the results shown in Table 2. The significant difference between groups 4 and 5 indicates that the mechanism underlying the effect may be different or that some feature of the treatment was more dominant in the latter case.

These results indicate that there is a significant effect of verteporfin-based PDT when used as a pretreatment adjuvant to radiation therapy when given as a single 10-Gy dose to the RIF-1 tumor. The tumor regrowth delay is increased by a factor that is more than additive, indicating a potential benefit for PDT when used as an adjuvant to radiation. This result is consistent with our central hypothesis that PDT can be used to increase the oxygen available in the tumor by suppressing the oxygen consumption of the remaining tumor. Also, when this non-vascular-targeting form of PDT is used as a pretreatment, it should enhance the radiation effect. It is also possible that the blood flow shunting that could occur as some microvessels are occluded may also cause oxygen enhancement in areas of continued flow, and pathological analysis and Doppler blood flow measurements are ongoing to examine this as a potential alternative hypothesis. Independent of the mechanism, combining PDT in a non-vascular-targeting regime (i.e., where there is a long latency between drug injection and the time of optical treatment) may be used effectively to enhance the efficacy of radiation therapy.

Experiments of combined therapies can often have potentially confounding causes, and it is important to not attribute the supra-additive effect observed here to a mechanism that cannot be directly proven. We hypothesize that a major mechanism causing this effect is the shift in tumor oxygenation toward higher PO2 and possibly spatially to reduce the preexisting hypoxic fraction within the tumor. One potentially confounding issue, which needs to be addressed, is the potential for hyperthermia induced by the high fluence rate of 133 mW/cm². Whereas some authors have indicated that this can induce hyperthermia when delivered at 633 nm, our previous measurements (16) have shown that the temperature rise with a higher fluence rate of 200 mW/cm² at 690 nm is 3–4 degrees C maximally and thus does not correspond to a hyperthermic rise, which requires 8–10 degrees C increase for a measurable effect on the tissue (51, 70). Nevertheless, it is possible that localized microscopic regions of the tumor experience hyperthermic rises in temperature. Nonetheless, there is a clear observation that combining PDT with radiation therapy simultaneously induces a greater than additive effect of tumor killing, and the mechanism remains to be conclusively delineated.

In summary, the cellular respiration of *in vitro* RIF-1 tumor cells can be significantly reduced in response to PDT treatment under the conditions examined here, and the dose response of this effect is identical to the results of a cell survival (MTS) assay. These results *in vitro* support the conclusion that this loss in cell metabolism is directly proportional to the loss of cell viability. The decrease in metabolism likely leads to a greater diffusion of oxygen throughout the remaining tissue *in vivo*, when the blood flow remains patent immediately after treatment. Experiments confirm that an increase in tumor tissue PO2 occurs after treatment and that the tumor tissue actually can rise above the radiobiologically hypoxic level. These observations, taken together, indicate a new direction and potentially a new set of applications for PDT in modulating the oxygenation of tissue.

Combining this type of PDT treatment with radiation treatment using a single 10-Gy dose of radiation demonstrated a greater than additive effect of tumor killing, as measured by tumor volume regrowth delay assay for the tumor doubling time. The mechanism for this synergistic effect may be due to the enhanced oxygen available during the treatment, effectively reducing the fraction of the tumor that is chronically hypoxic. Calculations of the oxygen change are in reasonable agreement with experimental observations. Additional experiments are required to help verify that this change in hypoxic fraction is the cause of the enhanced killing effect.

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Photodynamic Therapy with Verteporfin in the Radiation-induced Fibrosarcoma-1 Tumor Causes Enhanced Radiation Sensitivity

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