Tumor Vascular Response to Photodynamic Therapy and the Antivascular Agent 5,6-Dimethylxanthenone-4-Acetic Acid: Implications for Combination Therapy

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Abstract

Purpose: Photodynamic therapy (PDT) is a clinically approved treatment for a variety of solid malignancies. 5,6-Dimethylxanthenone-4-acetic acid (DMXAA) is a potent vascular targeting agent that has been shown to be effective against a variety of experimental rodent tumors and xenografts and is currently undergoing clinical evaluation. We have previously reported that the activity of PDT against transplanted mouse tumors is selectively enhanced by DMXAA. In the present study, we investigated the in vivo tumor vascular responses to the two treatments given alone and in combination.

Experimental Design: Vascular responses to (i) four different PDT regimens using the photosensitizer 2-[1-hexyloxyethyl]-2-devinyl pyropheophorbide-a (HPPH) at two different fluences (128 and 48 J/cm²) and fluence rates (112 and 14 mW/cm²), (ii) 5-aminolevulinic acid (ALA)–sensitized PDT (135 J/cm² at 75 mW/cm²), (iii) DMXAA at a high (30 mg/kg) and low dose (25 mg/kg), and (iv) the combination of HPPH-PDT (48 J/cm² at 112 mW/cm²) and low-dose DMXAA were studied in BALB/c mice bearing Colon-26 tumors.

Results: PDT-induced changes in vascular permeability, determined using noninvasive magnetic resonance imaging with a macromolecular contrast agent, were regimen dependent and did not predict tumor curability. However, a pattern of increasing (4 hours after treatment) and then decreasing (24 hours after) contrast agent concentrations in tumors, seen after high-dose DMXAA or the combination of PDT and low-dose DMXAA, was associated with long-term cure rates of >70%. This pattern was attributed to an initial increase in vessel permeability followed by substantial endothelial cell damage (CD31 immunohistochemistry) and loss of blood flow (fluorescein exclusion assay). Low dose – rate PDT, regardless of the delivered dose, increased the level of magnetic resonance contrast agent in peritumoral tissue, whereas treatment with either DMXAA alone, or PDT and DMXAA in combination resulted in a more selective tumor vascular response.

Conclusions: The observed temporal and spatial differences in the response of tumor vessels to PDT and DMXAA treatments could provide valuable assistance in the optimization of scheduling when combining these therapies. The combination of PDT and DMXAA provides therapeutically synergistic and selective antitumor activity. Clinical evaluation of this combination is warranted.

Therapies targeted towards the tumor vasculature offer effective strategies for controlling tumor growth. In combination with treatments aimed at direct cell kill, they provide greater opportunities to improve clinical outcome. Photodynamic therapy (PDT) is a clinically approved treatment for solid tumors and is based on the photoactivation of a tumor-localizing agent that results in the generation of cytotoxic singlet oxygen (1, 2). The tumor response to PDT is complex, involving vascular damage, direct tumor cell kill, and the induction of innate and adaptive immune responses. The relative contributions of each of these responses and the extent of antitumor activity depend on the PDT treatment regimen (3). Vascular events observed following PDT include release of vasoactive molecules, early, enhanced leakage, and platelet aggregation followed by occlusion (4–6). Over the years, PDT has been clinically effective as a curative (1) and palliative treatment (7) for a variety of malignant and nonmalignant diseases. Although the overwhelming majority of patients show a clinical response to PDT, some tumors will recur following treatment (1, 7), suggesting an advantage to using PDT in combination with other antitumor modalities.

5,6-Dimethylxanthenone-4-acetic acid (DMXAA) is a potent antivascular agent with selective tumor-targeting activity (8). DMXAA has been shown to induce vascular collapse and necrosis.
in murine tumors and xenografts (8, 9) and significant reductions in tumor blood flow in humans enrolled in phase I clinical trials (10). A wide variety of biological responses ranging from cytokine induction to activation of macrophage and natural killer cell activity have also been associated with DMXAA (11). The antitumor and antivascular effects of DMXAA seem mediated through a combination of direct effects of the drug on tumor vascular endothelial cells (12) and indirect effects through the induction of tumor necrosis factor-α (TNF-α; ref. 13), serotonin (14), IFN-inducible protein 10 (15), and nitric oxide (16).

We have previously reported that the exogenous administration of recombinant human TNF-α improved PDT activity against transplanted syngeneic mouse tumors without increasing normal tissue phototoxicity (17). This finding led to combination studies using PDT and DMXAA, because DMXAA is a potent and tumor-selective inducer of TNF-α. We reported recently that DMXAA significantly enhanced the antitumor activity of Photofrin-PDT in transplanted RIF-1 tumors without significant increases in dark- or photo-toxicity (18). Continuing studies are aimed at understanding the mechanistic similarities and differences between DMXAA and PDT to optimize protocol design and maximize the therapeutic efficacy of combination therapy. As a part of these investigations, magnetic resonance (MR) imaging was chosen as a noninvasive tool to define the in vivo tumor vascular response to PDT and DMXAA treatments when given alone and in combination. Specifically, vascular permeability was assessed in BALB/c mice with transplanted Colon-26 (murine colon carcinoma) tumors by using serial T1-weighted images following injection of a macromolecular contrast agent (19). Relaxation rates in tumor and normal tissue were measured before and after contrast agent injection and at different times following treatment. Vascular responses were evaluated following (i) PDT with 5-aminolevulinic acid (ALA); (ii) PDT with 2-[1-hexyloxyethyl]-2-devinyl pyropheophorbide-a (HPHPH) using a variety of treatment regimens that have been shown (3) to result in different patterns of early vascular, cellular and inflammatory responses, and various long-term tumor control rates; (iii) treatment with DMXAA at two different doses that result in either very low or high tumor cure rates; and (iv) treatment with a combination of DMXAA and PDT at doses that have minimal antitumor activities when given individually but together result in a high tumor cure rate (20). Vascular perfusion following PDT and DMXAA monotherapies and their combination was also evaluated using fluorescein dye exclusion. In addition, histologic and immunohistochemical studies were done to correlate with the MR findings and confirm vascular damage following treatment. The intensities and patterns of the vascular responses to PDT and DMXAA alone and in combination, and their relationship to tumor curability are discussed.

Materials and Methods

Chemicals. Clinical-grade HPPH (synthesized in our laboratory) was dissolved in water containing 5% dextrose (DSW), 2% ethanol, and 0.1% Tween 80 and was stored frozen (−4°C) in the dark. Solid DMXAA (provided by Gordon Newcalle, University of Auckland, New Zealand) was stored at room temperature in the dark. 5-ALA was obtained from Sigma Chemical Co. (St. Louis, MO).

Animals and tumor system. BALB/c-AnNCr mice were obtained from the Jackson Laboratory (Bar Harbor, ME). Animals were housed in microisolator cages in a laminar flow unit under ambient light. Eight- to 12-week-old animals were inoculated s.c. on the right shoulder with 10^5 Colon-26 (murine colon carcinoma) cells harvested from exponentially growing cultures. Tumors were used for experimentation 7 to 10 days after inoculation when they had grown to 6 to 8 mm in diameter. All the procedures were carried out according to protocols approved by the RPCI Institutional Animal Care and Use Committee.

Photodynamic therapy treatment. For in vivo PDT treatment, HPPH was diluted in HBSS containing 2% ethanol and 0.1% Tween 80 and injected i.t. via tail vein at a dose of 0.4 μmol/kg. Approximately 24 hours later, the tumors were illuminated with 665-nm wavelength light. The fur over each tumor was removed by shaving and depilation with Nair (Carter-Wallace, Inc., New York, NY) 48 hours before light treatment. Mice were immobilized in specially designed Plexiglas holders without anesthesia for light treatment. The light source consisted of a dye laser (Spectra-Physics, Mountain View, CA) pumped by an argon ion laser (Spectra-Physics) tuned to 665 nm using DCM dye (Exciton, Dayton, OH). Multiple animals were treated simultaneously by passing the output from the dye laser through an eight-way beam splitter (21). A spot diameter of 1.1 cm was used for all of the treatments. Four illumination regimes were used for HPPH-sensitized PDT, with fluence and fluence rates of 48 J/cm^2 delivered at 14 mW/cm^2, 48 J/cm^2 at 112 mW/cm^2, 128 J/cm^2 at 14 mW/cm^2, and 128 J/cm^2 at 112 mW/cm^2. For topical PDT using ALA, six applications (20 μl per each application, 15 minutes apart) of 20% ALA in a proprietary vehicle (DUSA Pharmaceuticals, Inc., Wilmington, MA) were applied onto the depilated tumor surface using a sterile applicator tip 3 hours before light exposure. Care was taken to remove the excess photosensitizer from the normal skin surrounding the tumor. The animals were given an i.p. injection of ketamine/xylazine mixture (10:1) at a dose of 1.0 ml/100 g to alleviate any discomfort or pain during the light treatment. The same laser setup used for HPPH-PDT was used for topical ALA-PDT. Following topical ALA application, tumors were irradiated locally with 635-nm light at a fluence of 135 J/cm^2 delivered at a fluence rate of 75 mW/cm^2 over an area 1.1 cm in diameter.

5.6-Dimethylxanthenone-4-acetic acid treatment. DMXAA was freshly dissolved in 5% sodium bicarbonate immediately before use. BALB/c mice bearing Colon-26 tumors were injected i.p. with either 25 or 30 mg/kg DMXAA.

Photodynamic therapy/5,6-dimethylxanthenone-4-acetic acid combination treatment. Combination studies were carried out using an HPPH dose of 0.4 μmol/kg injected 24 hours before light illumination at a wavelength of 665 nm (48 J/cm^2 at 112 mW/cm^2). DMXAA was injected at a dose of 25 mg/kg 2 hours before light treatment.

Immunohistochemical analyses. Tumors were harvested at different times after treatments and placed immediately in Tris-buffered zinc fixative [0.1 mol/L Tris-HCl buffer (pH 7.4) containing 3.2 mmol/L calcium acetate, 22.8 mmol/L zinc acetate, and 36.7 mmol/L zinc chloride] for 18 hours, transferred to 70% ethanol, dehydrated, and embedded in paraffin. Five-μm-thick sections were stained by routine immunohistochemical methods using peroxidase neutralization. Slides were counterstained with Harris hematoxylin (Poly Scientific, Bayshore, NY). Mouse CD31 was detected with a rat monoclonal antibody (IgG2a; PharMingen, San Diego, CA) at 1:50 dilution in PBS for 60 minutes at 37°C followed by biotinylated rabbit anti-rat immunoglobulin G (12112D; PharMingen) at 1:100 dilution for 30 minutes, streptavidin peroxidase (50-242; Zymed, San Francisco, CA) for 30 minutes, and diamobenzidine for 5 minutes.

Fluorescein dye exclusion assay. Tumor vascular damage following PDT and DMXAA, alone and in combination, was examined using a fluorescein exclusion assay (22). Briefly, BALB/c mice bearing Colon-26 tumors were injected i.v. (retro-orbital route) with 20 mg/kg fluorescein (Sigma Chemical) either 4 or 24 hours after treatment. The animals were euthanized after 2 minutes and the tumors were removed, weighed, and dissolved overnight in Solvable (Packard Instrument Co., Meriden, CT) tissue solubilizer. Fluorescein exclusion was calculated as:

\[ 1 - \frac{F_{\text{treated}}}{F_{\text{control}}} \]
where $F_{\text{treated}}$ and $F_{\text{control}}$ were the concentrations of fluorescein found in treated and untreated tumors, respectively. Concentrations were estimated by measuring fluorescein fluorescence with a FluoroMax-2 (Jobin Yvon/Spekex, Edison, NJ) fluorimeter as described previously (22).

**Magnetic resonance contrast-enhancing agent.** MacroGd (methoxy-PEG succinyl-poly-L-lysine-GdDTPA) was purchased from Pharmalan Ltd. (Buffalo Grove, IL). The agent has been reported to have an average molecular weight of 530 kDa, a hydrodynamic diameter of ~10 nm (19) and consists of a monomethoxy ether of poly(ethylene glycol) covalently attached to poly(L-lysine), with poly(L-lysine) serving as the carrier of Gd-DTPA. Before injection, 100 mg of lyophilized powder of MacroGd was dissolved in 2 mL of sterile saline at room temperature and prewarmed to 37°C. The solution was injected via tail vein into mice before treatment (PDT or DMXAA). The injection volume was based on recommendations by the manufacturer.

**Magnetic resonance acquisition.** MR imaging was done 7 to 10 days after implantation of Colon-26 cells, when tumors were ~6 to 8 mm in diameter. Before the acquisitions, mice were anesthetized with 4% isoflurane (Abbott Laboratories, Chicago, IL) and anesthesia was maintained at 2% isoflurane during scanning by means of an inlet tube. The animals were secured in a mouse coil chamber and positioned in the scanner. Animal body temperature in the scanner was maintained at 37°C by a circulating water bath. MR images were acquired using a 4.7 T/33-cm horizontal bore magnet (GE NMR Instruments, Fremont, CA) incorporating AVANCE digital electronics (Bruker Biospec with ParaVision 2.1; Bruker Medical, Billerica, MA) and a removable gradient coil insert (G060, Bruker Medical) generating maximum field strength of 950 mT/m and a custom-designed 35-mm radiofrequency transceiver coil. Data acquisition consisted of a localizer, T1-weighted and T2-weighted images. T2-weighted spin-echo sequences with an effective time of echo period (TE) = 10 ms and a flip angle = 25 degrees, number of averages = 3. Whole-body angiography was acquired using a three-dimensional spoiled gradient recalled echo scan (matrix size = 192 × 128 × 128, field of view = 48 × 32 × 32 mm, TE = 3.0 milliseconds, TR = 15 milliseconds, flip angle = 25 degrees, number of averages = 2).

**Magnetic resonance data analysis.** Before in vivo experiments, in vitro studies were done to determine the relationship between the contrast agent concentration and relaxation rate. Following administration of the contrast agent, a three-dimensional spoiled gradient echo sequence was acquired (matrix size = 32 × 32 × 32 mm, slice thickness = 1.0 mm, matrix size = 128 × 96 voxels, number of averages = 3). Whole-body angiography was acquired using a three-dimensional spoiled gradient recalled echo scan (matrix size = 192 × 128 × 128, field of view = 48 × 32 × 32 mm, TE = 3.0 milliseconds, TR = 15 milliseconds, flip angle = 25 degrees, number of averages = 2).

The relaxation rate of the contrast agent depends on the concentration of fluorescein in tumor tissue. The relaxation rate of the tumor ($R_1$) was determined to be 3.373 (s/cm/mmol/L)−1 with a correlation coefficient of $R^2 = 0.98$, by linear least-squares analysis. Image analysis and three-dimensional renderings were done using commercially available software (AnalyzePC version 3.0; Biomedical Imaging Resource, Mayo Foundation, Rochester, MN). Raw data were reformatted and object maps of desired regions of interest were outlined. Signal intensities from regions of interest were obtained and mean intensity within the regions of interest was used for calculating the T1 relaxation at each TR time. The relaxation rate $R_1$ and the maximal signal intensity $S_{\text{max}}$ were then obtained by nonlinear fitting of the equation, using Matlab’s curve-fitting toolbox (Matlab 6.5; Mathworks, Inc., Natick, MA), following subtraction of background noise:

$$S_{\text{TR}} = S_{\text{max}}(1 - e^{-(R_1 \times \text{TR})})$$

Where $S_{\text{TR}}$ is the signal intensity obtained at each TR time. Three-dimensional renderings were obtained by maximum intensity projection algorithm (Analyze PC version 3.0; Biomedical Imaging Resource) for visualization of vascular permeability subsequent to contrast agent administration. T1 relaxation maps of animals were also calculated on a pixel-by-pixel basis to assess vascular permeability in response to treatments.

**Statistical analyses.** All measured values are reported as means ± SE. The two-tailed $t$ test was used for comparing the individual treatment groups with the controls at different times. $P < 0.05$ was considered statistically significant. All statistical calculations and analyses were done using GraphPad Instat (ver. 3.00, GraphPad Software, San Diego, CA).

**Results**

**Tumor vascular response to photodynamic therapy.** Systemic PDT was carried out using HPPH with four different treatment regimens that were shown previously to induce dissimilar patterns of cellular, immune and vascular responses (3); the long-term tumor control levels for these regimens range from 0% to 70% and are listed in Table 1. Changes in vascular permeability in response to PDT were assessed using contrast-enhanced MR imaging by calculating and comparing the relaxation rates in the tumor before and after treatment (Fig. 1A). Three-dimensional renderings by maximum intensity projection were also created to visualize changes in vascular permeability following treatment (Fig. 2). Among the four HPPH-PDT regimens used in our study, the low-fluence and low fluence rate regimen (48 J/cm$^2$ delivered at 14 mW/cm$^2$; 10–20% 90-day cure rate) showed the largest increase in tumor vessel permeability over untreated controls ($P < 0.05$ at 4 hours; $P < 0.01$ at 24 hours) as shown in Fig. 1A, b. Figure 2A, f-j shows the corresponding maximum intensity projection images for this regimen. Notably, this regimen resulted also in a diffuse pattern of vascular leakiness in the peritumoral tissue at 24 hours, as shown by the increase in signal intensity in the region outside the tumor-bounding box drawn in Fig. 2A, f. T1 relaxation maps calculated on a pixel-by-pixel basis on these animals also confirmed the extensive leakage of the contrast agent within the tumor tissue and in the surrounding normal tissues (Fig. 3b-c) at both time points after treatment. Animals treated with the PDT regimen using 128 J/cm$^2$ at 14 mW/cm$^2$ resulted in a similar diffuse pattern of leakiness around the tumor (Figs. 2C, c and 3, e-f), although the change in intratumoral vascular permeability was not statistically significant compared with the controls ($P > 0.05$ at 4 and 24 hours; Fig. 1A, c). The regimen using 128 J/cm$^2$ at 112 mW/cm$^2$ (6-10% 90-day cure rate), although associated with a tumor cure rate similar to that following the PDT regimen with 48 J/cm$^2$ at 14 mW/cm$^2$, did not induce a comparable increase in vascular permeability (Fig. 1A, b). Finally, the systemic PDT regimen using 48 J/cm$^2$ at 112 mW/cm$^2$ produced no long-term tumor control and the vascular response to this regimen was also minimal ($P = 0.05$ at 4 hours, $P > 0.05$ at 24 hours versus control; Fig. 1A, a).

Topical PDT was patterned after a regimen used in the clinical treatment of cutaneous tumors (20% ALA: 135 J/cm$^2$ at a rate of 75 mW/cm$^2$; 0% 90-day cures: Table 1). This treatment
regimen produced a small but statistically significant increase in the tumor relaxation rate only at 4 hours after illumination (Fig. 1A, a; *P < 0.05 versus control).

To evaluate the effects of the different HPPH-PDT regimens on tumor vascular perfusion, we used a fluorescein exclusion assay (22). The ineffective (no tumor cures) PDT regimen (48 J/cm² delivered at 112 mW/cm²) did not cause a significant change in perfusion (*P > 0.05 versus control at 24 hours; Fig. 4a). The PDT regimens (128 J/cm² at 112 mW/cm² and 48 J/cm² at 14 mW/cm²) that result in similar intermediate long-term cure rates (6-20%) produced moderate reductions in perfusion (~65% at 24 hours after light; Fig. 4b). Treatment with the most effective PDT regimen (128 J/cm² at 14 mW/cm²; 60-75% 90-day cures) resulted in a >90% reduction (*P < 0.001 versus control) in vascular perfusion 24 hours after treatment (Fig. 4c).

**Tumor vascular response to 5,6-dimethylxanthenone-4-acetic acid.** Vascular responses were studied using contrast-enhanced MR imaging following administration of either 25 or 30 mg/kg DMXAA. The images were acquired before contrast agent administration, after contrast agent administration before DMXAA injection and at 4 and 24 hours after DMXAA. A significant (*P < 0.01) increase in vascular permeability was observed 4 hours after treatment with the high, tumor-curative (70% 90-day cures, Table 1) DMXAA dose (30 mg/kg) compared with the untreated controls (Fig. 1A, c). Conversely, after 24 hours, there was a significant decrease in the tumor relaxation rate relative to the controls (*P < 0.05); this was likely due to a reduction in available circulating contrast agent resulting from the DMXAA-induced disruption of tumor vessels, as shown by the virtual absence of CD31-immunostained endothelial cells and extensive hemorrhage (Fig. 5). This was confirmed by the fluorescein exclusion assay, which indicated an ~85% reduction in vascular perfusion at 24 hours (Fig. 4c; *P < 0.001 versus control). In contrast to PDT, the vascular response induced by 30 mg/kg DMXAA was relatively confined to the tumor as seen in the maximum intensity projection images (Fig. 2C, d-f) and the T1 relaxation maps (Fig. 3g-i). DMXAA is a tumor-selective antivascular, antitumor agent (11). This selectivity is shown by the marked increase in MR contrast agent in the tumor relative to muscle at 4 hours after drug administration (Fig. 1B). The reduction in tumor-to-muscle ratio to a value below that for control mice reflects the loss of functional vessels in the tumor between 4 and 24 hours. Mice treated with the low, subcurative dose (25 mg/kg; 5% 90-day cures) showed no increase in permeability in the tumor or surrounding tissue relative to untreated controls (Fig. 1A, a and Fig. 2B, g-i). Histologic sections of these tumors showed preservation of CD31 immunostaining of the vascular endothelium and little apparent hemorrhage (Fig. 5). The fluorescein exclusion data (Fig. 4a) suggests only a small decrease in tumor vascular perfusion at 24 hours after low-dose DMXAA (25 mg/kg), although this was not statistically significant (*P > 0.05 versus control).

**Effect of tumor-curative photodynamic therapy/5,6-dimethylxanthenone-4-acetic acid combination therapy on vasculature.** We have previously shown that low-dose DMXAA (25 mg/kg) markedly enhances Colon-26 tumor response to subcurative and noncurative HPPH-PDT regimens (20). In the present study, we combined 25 mg/kg DMXAA (5% 90-day cures as a single therapy, Table 1) and the HPPH-PDT regimen 48 J/cm² at 112 mW/cm² (0% 90-day cures as a single therapy), resulting in >70% long-term cures. This combination showed an increase in permeability at 4 hours over those obtained with the PDT regimen alone, DMXAA alone, or the untreated controls (Fig. 1A). There was a significant decrease in the tumor relaxation rates (Fig. 1A, c and B) 24 hours after treatment with the PDT-DMXAA combination (*P < 0.01 versus control; *P < 0.01 versus 4

### Table 1. T1 relaxation rates in Colon-26 tumors following treatment with DMXAA, HPPH-, or ALA-mediated photodynamic therapy, or the combination of DMXAA and HPPH-PDT

<table>
<thead>
<tr>
<th>Treatment*</th>
<th>PDT treatment duration</th>
<th>Tumor T1 relaxation rates (Hz)</th>
<th>Percent 90-d cures of Colon-26 tumors</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(hh:mm:ss)</td>
<td>Before treatment</td>
<td>4 h after treatment</td>
</tr>
<tr>
<td>Control</td>
<td></td>
<td>0.553 ± 0.018</td>
<td>0.765 ± 0.046</td>
</tr>
<tr>
<td>DMXAA 25 mg/kg</td>
<td></td>
<td>0.555 ± 0.011</td>
<td>0.716 ± 0.059</td>
</tr>
<tr>
<td>DMXAA 30 mg/kg</td>
<td></td>
<td>0.543 ± 0.032</td>
<td>1.391 ± 0.105†</td>
</tr>
<tr>
<td>HPPH-PDT 128 at 112</td>
<td></td>
<td>0.724 ± 0.053</td>
<td>1.396 ± 0.097</td>
</tr>
<tr>
<td>HPPH-PDT 128 at 14</td>
<td></td>
<td>0.543 ± 0.024</td>
<td>0.790 ± 0.044</td>
</tr>
<tr>
<td>HPPH-PDT 48 at 14</td>
<td></td>
<td>0.598 ± 0.032</td>
<td>1.125 ± 0.096†</td>
</tr>
<tr>
<td>HPPH-PDT 48 at 112</td>
<td></td>
<td>0.585 ± 0.006</td>
<td>0.923 ± 0.038</td>
</tr>
<tr>
<td>ALA-PDT 135 at 75</td>
<td></td>
<td>0.599 ± 0.016</td>
<td>0.964 ± 0.026†</td>
</tr>
<tr>
<td>Combination DMXAA 25 mg/kg + HPPH-PDT 48 at 112</td>
<td></td>
<td>0.549 ± 0.020</td>
<td>1.100 ± 0.045†</td>
</tr>
</tbody>
</table>

*HPPH-PDT and ALA-PDT treatments used a fixed photosensitizer dose; however, the drug-activating light was delivered at different doses (fluences) and dose rates (fluence rates), represented as “fluence (J/cm²) delivered at fluence rate (mW/cm²)”.
†Bellnier et al. (20).
‡P < 0.01, two-tailed t test.
§P > 0.05, two-tailed t test.
‖Henderson et al. (3).
*Unpublished.
**DMXAA was injected 2 hours before the beginning of PDT light delivery.
Vascular Response to Photodynamic Therapy and DMXAA

Discussion

Combining therapies with antitumor activities and nonoverlapping toxic effects can be a useful strategy for improving treatment outcome (23). We have previously shown that the antitumor drug DMXAA enhances the activity of Photofrin-sensitized PDT against experimental RIF-1 tumors without increasing systemic toxicity or local phototoxicity (18). DMXAA has potent effects on tumor vasculature: although PDT causes direct tumor cytotoxicity and can induce immune responses, antitumor activity strongly depends on its vascular-damaging effects. As a part of our combination therapy studies, we were interested in evaluating the mechanistic differences in vascular responses between PDT and DMXAA and their combination in the Colon-26 mouse model. To address this we assessed the effects of PDT using two clinically relevant photosensitizers (systemic HPPH and topical ALA, which leads to the biosynthesis of the photosensitizer protoporphyrin IX) and DMXAA, alone and in combination with HPPH-PDT, using noninvasive MR imaging with a macromolecular contrast-enhancing agent (19).

The results clearly show that (i) both PDT and DMXAA can significantly enhance the permeability of tumors when given as single agents; (ii) changes in vascular permeability following DMXAA, and DMXAA combined with PDT, were largely confined to the tumor whereas some treatments with PDT increased permeability in both the tumor and peritumoral tissue; and (iii) reductions in vascular perfusion seem more predictive of treatment outcome than changes in vascular permeability.

PDT regimens using high fluences (>100 J/cm²) and high fluence rates (~150 mW/cm²) have become the standard in clinical therapy. However, high fluence rates can lead to rapid consumption of the ambient molecular oxygen required for maximum antitumor activity (ref. 3 and citations therein). For example, a light dose of 128 J/cm² delivered to Colon-26 tumors (in BALB/c mice given HPPH) at fluence rates of 112 and 14 mW/cm² resulted in 90-day cures of 5% to 10% and 60% to 75%, respectively (3). In addition, the treatment regimen plays a key role in the pattern of PDT damage. In the report of Henderson et al. (3) low fluence rate PDT (14 mW/cm²) induced higher levels of apoptosis than high fluence rate (112 mW/cm²) PDT. Moreover, high fluence (128 J/cm²) at the low fluence rate led to ablation of CD31-stained vascular endothelial cells, whereas the same fluence at a high fluence rate had no effect on CD31 immunostaining of the endothelium. We, therefore, chose to examine these same HPPH-PDT regimens in our MR imaging/vascular response study. DMXAA is a well-known vascular-targeting agent that causes rapid, dose-dependent antitumor activity in rodent models. For this study, we employed two DMXAA doses: a low (25 mg/kg) dose with minimal antitumor activity that we often use in our PDT-DMXAA combination studies and a high (30 mg/kg) dose with very good antitumor activity (ref. 20; Table 1). Finally, we are also exploring the combination of ALA-based PDT plus DMXAA (24); for the work presented here, we used a PDT regimen that closely approximates the clinical standard.

Malignant tumors are characterized by malformed and leaky vessels (25), accounting for the increased accumulation of contrast agent over time in the untreated Colon-26 tumors. For this reason, we used time-matched controls in the MR imaging experiments as shown in Fig. 1A. Among the HPPH-PDT treatments, the regimen that employed the lowest fluence and lowest fluence rate (48 J/cm² at 14 mW/cm²; Fig. 1A, b) showed the greatest increase in vascular permeability. This regimen is associated with minimal antitumor activity (Table 1). In sharp contrast, the regimen 128 J/cm² at 14 mW/cm², which is

![Fig. 1. A. summary of the vascular responses of Colon-26 tumors to PDT, DMXAA, and PDT-DMXAA combination therapy. Vascular permeability was estimated by measuring the tumor T1 relaxation rates before and 4 and 24 hours after treatment. Responses are grouped according to the long-term tumor control rates, listed in Table 1, as follows: (a) 0% to 5%, (b) 6% to 20%, (c) 60% to 75% 90-day cures. PDT treatment regimens are represented as "fluence (J/cm²)" delivered at (cm²) fluence rate (mW/cm²); for example, "48@112" refers to a fluence of 48 J/cm² delivered at a fluence rate of 112 mW/cm². HPPH was injected i.v. at a dose of 0.4 μmol/kg ~ 24 hours before application of the activating laser light; ALA was applied topically using six applications, 20 μL per each application, of 20% ALA 3 hours before light exposure. Columns and points, mean from three to four mice per group; bars, ± SE. B. tumor-to-muscle ratios of MR relaxation rates of untreated controls (C) and animals treated with 25 mg/kg DMXAA (m), 30 mg/kg DMXAA (i), HPPH-PDT using the regimen 48@112 (.), and the combination of 25 mg/kg DMXAA injected 2 hours before HPPH-PDT using the regimen 48@112 (•).](www.aacrjournals.org)
Fig. 2. A, tissue vascular responses, represented by whole body renderings created by maximum intensity projection using three-dimensional spoiled gradient echo MR scans. Images a, d, and g were acquired before treatment; images b, e, and h were acquired 4 hours after treatment; and images c, f, and i were acquired 24 hours after treatment. Images a - c are for control tumors; images d - f are for treatment with the HPPH-PDT regimen 48@14; images g - i are for the HPPH-PDT regimen 128@112. B, maximum intensity projection images a - c are for treatment with the HPPH-PDT regimen 48@112; images d - f are for treatment with ALA-PDT using the regimen 135@75; images g - i are for treatment with 25 mg/kg DMXAA.
associated with high long-term cure rates in this model, did not cause a significant increase in permeability over time. However, the marked reduction in tumor tissue perfusion observed at 24 hours following treatment (Fig. 4c) is suggestive of other vascular mechanisms involved in the antitumor PDT response.

The findings of our study are in agreement with the report of Snyder et al. (26), who studied the permeabilizing effects of HPPH-PDT using fluorescent microspheres of different diameters. In that study, the leakage of fluorescent beads into Colon-26 tumors was greatest following regimens using fluences of 48 to 88 J/cm² and fluence rates of 14 to 28 mW/cm². The escape of liposome-encapsulated doxorubicin (Doxil) was also significantly enhanced by low-dose, low dose rate HPPH-PDT, resulting in increased tumor control (26). Our MR images (e.g., the T1 relaxation maps in Fig. 3) show that PDT-induced changes in vascular permeability affected not only the Colon-26 tumor but also tissue surrounding the tumor, an issue not addressed in the microsphere/Doxil study (26). Visual inspection also revealed more edema around the tumor in these animals after treatment.

Topical ALA-based PDT is a widely used clinical treatment modality for malignant and nonmalignant cutaneous lesions. However, the mechanisms involved in tumor destruction following ALA-PDT are not clear. In particular, the effects of ALA-PDT on the vasculature and its contribution to the long-term tumor response are controversial (27–30). Tumor vessel permeability increased only slightly over the time-matched control value following topical ALA-PDT, and this was seen only at the 4-hour interval after light exposure. It should be noted that this treatment regimen (20% topical ALA, 135 J/cm² at 75 mW/cm²) results in only a 1-week delay in Colon-26 tumor growth and no long-term cures (Table 1). Regardless, the limited change in vessel permeability coupled with no alteration in CD31 immunostaining (24) after 24 hours suggest that nonvascular mechanisms dominate the response of this tumor to ALA-PDT.

Tumor vascular response to PDT, which is strongly dependent on the choice of photosensitizer and treatment conditions, can include early changes in permeability, vessel constriction, and platelet aggregation (4, 5). Although the precise mechanism behind PDT-induced vascular permeability is not known, it is believed to be due to the rapid formation of endothelial gaps (4–6). Tumor response to HPPH-PDT does not involve vascular damage alone, but it plays a significant role in achieving long-term tumor control (3). Changes in vascular permeability following PDT did not serve as a predictive marker of tumor response to treatment. However, the amount of reduction in vascular perfusion following treatment seemed to have a strong correlation with the long-term cure rates seen in our tumor model.

The effects of DMXAA on tumor microcirculation have recently been studied using dynamic contrast enhanced MR imaging in clinical studies (10). The increase in permeability seen following DMXAA administration is believed to be one of the earliest possible effects of the drug that has a direct effect on

**Fig. 2** Continued. C, maximum intensity projection images a–c are for treatment with the HPPH-PDT regimen 128@14; images d–f are for treatment with 30 mg/kg DMXAA; images g–i are for treatment with the combination of 25 mg/kg DMXAA and the HPPH-PDT regimen 48@112. The locations of the implanted Colon-26 tumors are illustrated with the help of the yellow bounding boxes. See Fig. 1 legend for an explanation of the PDT regimens.
interstitial pressure differences, compromising blood flow in tumor tissue (11). The vascular effects of DMXAA are believed to be the result of both direct drug effects on endothelial cells (12) and indirect effects mediated by the induction of TNF-α, serotonin, IFN-inducible protein 10, and nitric oxide (13–16). TNF-α has been shown to cause a permeability change across endothelial cell monolayers (31). Aicher et al. (32) have previously reported the use of a macromolecular MR contrast-enhancing agent to assess tumor vascular permeability following treatment of murine tumors with human recombinant TNF-α. More recently, the susceptibility of tumors to the vascular-targeting agent CA4P has been reported to correlate with vascular permeability (33). The results seen in our tumor model also show a steep dose-response curve for DMXAA with a high threshold, as reported previously by Lash et al. (34) and Siim et al. (35). In addition, changes in vascular permeability also show a dose dependency.

In our study, high-dose DMXAA (30 mg/kg) resulted in increased tumor vessel permeability after 4 hours but subsequently lead to a decrease at 24 hours relative to untreated, time-matched controls. This observation was not surprising, as high dose DMXAA has previously been shown to cause widespread tumor tissue necrosis with only a few islands of viable tissue remaining at ~24 hours after treatment (36). Our histopathologic studies also confirmed vessel damage, hemorrhage, and necrosis at the later time point. Fluorescein exclusion also revealed a drastic reduction in perfusion after high-dose DMXAA treatment. Furthermore, upon visual inspection of tumors the day after treatment with high-dose DMXAA, a well-circumscribed area of necrosis and hemorrhage was observed. In contrast, tumors treated with PDT often show extensive scabbing not only over the tumor but also the surrounding normal skin in the illumination field.
The disruption of blood flow induced by DMXAA alone seems insufficient to produce tumor growth delays in patients (37). As such, clinical trials combining DMXAA and agents such as paclitaxel and carboplatin in non–small cell lung carcinoma have recently commenced5 and combination studies of DMXAA with PDT are currently being planned by our group. Studies in preclinical models have shown the potential for DMXAA in combination with melphalan, radiotherapy, radioimmunotherapy, and hyperthermia (38). The enhanced vascular permeability following DMXAA may also escalate the delivery of chemotherapeutic agents to the tumor thereby offering an added advantage to combination strategies.

We have previously reported the enhancement of PDT by low-dose DMXAA against RIF-1 tumors (18) and Colon-26 tumors (20) in mice. For our present studies in Colon-26 tumors, we chose individual doses of PDT (48 J/cm² at 112 mW/cm²) and DMXAA (25 mg/kg) that had virtually no antitumor activity and no effects on the tumor vasculature. However, the combination of PDT and DMXAA at these ineffective doses seemed to have significant vascular damaging effects (MR data, fluorescein exclusion, and immunohistochemistry). At 24 hours after treatment, there was a significant decrease in the tumor T1 relaxation rates in animals treated with the combination regimen, which suggests the loss of functional vessels. CD31 staining of these tumors showed evidence of vascular damage. A significant reduction in tumor tissue perfusion was also seen following combination treatment as determined by fluorescein exclusion. The similar patterns of changes in permeability at the DMXAA dose of 30 mg/kg and PDT-DMXAA combination therapy at a DMXAA dose of 25 mg/kg with the PDT treatment regimen 48 J/cm² at

more, the PDT regimen (48 J/cm² at 112 mW/cm²) used for combination offers the advantage of a clinically feasible alternative to the much longer treatment time required with the PDT regimen (128 J/cm² at 14 mW/cm²; see Table 1 for treatment times) to achieve a comparable tumor response to these treatments. Further studies are now being done to establish and correlate changes in the vascular permeability and shutdown with the eventual tumor response to these treatments.

In summary, we have revealed differences in the response of tumor vessels to two treatments that have strong antivascular components, given as monotherapies and in combination. Taken together, the MR images, CD31 immunostaining, fluorochrome exclusion results, and the tumor control data suggest that changes in vessel permeability following PDT is a subtle injury and below the threshold needed to achieve the catastrophic vascular collapse and dissolution that leads to tumor destruction. This threshold can be crossed by increasing the PDT dose or by combining PDT with the vascular-damaging agent DMXAA. In addition, it seems that DMXAA also can enhance the selectivity of the tumor response to PDT. Finally, we have shown the potential for MR imaging using macromolecular contrast agents to aid in defining the threshold for vascular damage. Further preclinical evaluation of combination therapies using such noninvasive imaging techniques is important. Such studies will provide mechanistic insights into the interactions between the individual treatments and allow for optimization of scheduling for maximal therapeutic benefit.

References

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