Treatment with the Tumor Necrosis Factor-α-Inducing Drug 5,6-Dimethylxanthenone-4-Acetic Acid Enhances the Antitumor Activity of the Photodynamic Therapy of RIF-1 Mouse Tumors

David A. Bellnier, Sandra O. Gollnick, Susan H. Camacho, William R. Greco, and Richard T. Cheney

The Photodynamic Therapy Center, Department of Biostatistics, and Department of Pathology and Laboratory Medicine, Roswell Park Cancer Institute, Buffalo, New York

Abstract

DMXAA (5,6-dimethylxanthenone-4-acetic acid) is an antivascular agent that exerts its antitumor effect at least partly through the induction of tumor necrosis factor (TNF-α). Photodynamic therapy (PDT), the activation of a photoreactive drug in tumor tissue with visible light, is used clinically to control solid malignancies. PDT has been shown previously to be potentiated, in mice, by the i.p. administration of recombinant human TNF-α. Here, we investigated the activity of DMXAA as a modifier of Photofrin-based PDT of implanted murine RIF-1 tumors. The DMXAA dose (20 mg kg⁻¹) used throughout this study had little effect on tumor growth. The combination of DMXAA and PDT led to a reduction in tumor volume and significant delays in regrowth, giving a PDT-dose modification factor of 2.81. This enhancement was found to be strongly schedule dependent. The most pronounced responses were achieved when DMXAA was administered 1–3 h before the local illumination of the tumors; less activity was observed at other intervals within ±24 h of PDT-light delivery. Using a 2-h DMXAA-light interval, histological examination showed significantly reduced blood vessel counts (CD31 immunostaining) and marked necrosis (H&E) in the tumors given combination therapy compared with the tumors given either agent alone. Conversely, peritumoral tissue was still intact 24 h after the combined therapy. DMXAA did not augment the damage to normal mouse feet after low-dose PDT (1.5 mg kg⁻¹ Photofrin); however, there was some enhancement of normal tissue phototoxicity when DMXAA was combined with high-dose PDT. The antitumor effect after DMXAA plus low-dose PDT (1.5 mg kg⁻¹ Photofrin) appeared to be dependent on TNF-α because neutralizing antibodies to this cytokine reduced the tumor response to control levels. DMXAA by itself induced TNF-α in RIF-1 tumors whereas PDT did not. However, the addition of PDT after DMXAA resulted in decreases in TNF-α, suggesting that the enhanced antitumor activity of the combination therapy was not attributable simply to an increased induction of the cytokine by PDT over that from DMXAA alone. These observations suggest a promising new combination therapy with considerable therapeutic advantage.

Introduction

PDT using the sensitizer Photofrin (porfimer sodium) has been approved by the Food and Drug Administration for treatment of both early- and late-stage lung cancers and obstructive cancers of the esophagus in the United States and has received regulatory approval in Canada, Europe, and Japan for the treatment of these and other solid tumors (1). Photofrin can preferentially localize in many malignancies, and subsequent illumination with visible light results in the generation of tissue-damaging singlet oxygen (2). The antitumor response involves a complex interaction between direct cytotoxicity, secondary cell death as a result of vascular collapse and reduced blood flow, and a prominent inflammatory reaction (3).

In a previous study, we found that the combination of Photofrin-sensitized PDT and recombinant human TNF-α provided at least additive antitumor efficacy against an implanted mouse tumor (4). In addition, recombinant human TNF-α had little effect on normal tissue phototoxicity, indicating a positive therapeutic gain.

TNF-α has broad activity against solid murine tumors and xenografts. Tumor regression after TNF-α administration results largely from the inhibition of blood flow and the ensuing ischemia (5) and inflammation (6, 7). However, because TNF-α shows significant systemic toxicity at effective doses, its clinical use is restricted to regionally directed therapy such as isolated limb perfusion of patients with melanoma and sarcoma of the extremities (8), isolated hepatic perfusion of patients with nonresectable hepatic tumors (9), or intra-tumoral injections of a variety of malignancies (10). Attempts to limit TNF-α toxicity also include the systemic injection of agents that stimulate the synthesis of this cytokine preferentially in tumors. For example, FAA was proved to be a potent agent against a variety of solid experimental tumors, and its activity appears to be dependent on the induction of TNF-α (11). Unfortunately, although FAA-associated toxicity is generally milder than that from systemic TNF-α, this agent is also clinically inactive (12).

DMXAA is a structural analogue of FAA that demonstrates excellent activity against murine tumors (13). Similar to FAA, DMXAA antitumor activity depends, to a great part, on the induction of TNF-α (14) and results in tumor hemorrhagic necrosis. Additionally, DMXAA shows immune-modulatory effects, including induction of other cytokines and chemokines and stimulation of macrophage and natural killer cell activity (15), which may influence both short-term response and long-term control of tumors. DMXAA is in Phase 1 trials and shows evidence of clinical activity (16).

We evaluated the therapeutic effect of PDT in combination with DMXAA against the RIF-1 tumor implanted s.c. in mice. Because exogenous TNF-α administration was shown previously to enhance PDT antitumor activity in mice (4), we hypothesized that a combination therapy with DMXAA, which induces TNF-α synthesis in tumors, together with PDT might result in a potent and selective therapy. Here we have selected a DMXAA dose sufficient to induce TNF-α in tumors, but without any observable antitumor activity, and show for the first time that DMXAA selectively enhances Photofrin-sensitized PDT.
Materials and Methods

Materials. Photofrin (porfimer sodium) was obtained from Axcan Scandinapharm, Inc. (Birmingham, AL). The solid material was reconstituted with 5% D5W to a concentration of 2.5 mg/ml and stored at −70°C. Aliquots were thawed and diluted further with D5W immediately before use. Solid DMXAA was provided by Gordon Newcasttle (University of Auckland, Auckland, New Zealand) and formulated in 5% sodium bicarbonate immediately before injection. Neutralizing anti-mouse TNF-α monoclonal antibody (rat IgG1, clone MP6-XT3; BD Pharmingen, San Diego, CA) was diluted with D5W immediately before injection. Fluorescein (J. T. Baker Chemical Co., Phillipsburg, NJ) was dissolved in PBS (pH 7.2) to a concentration of 2 mg/ml.

Tumor Cells. The RIF-1 tumor cell line (17) was used in this study (radiation-induced fibrosarcoma, syngeneic to C3H/HeJ mice). The cells were maintained with 10% fetal bovine serum, 200 U/ml l-glutamine, 1% penicillin-streptomycin-neomycin in MEM-α medium (all components were purchased from Invitrogen Life Technologies, Inc., Carlsbad, CA); incubation conditions were 37°C and 5% CO2.

Cytokine Measurements. TNF-α protein expression in DMXAA, PDT, and DMXAA + PDT-treated mice was determined using an ELISA kit purchased from R&D Systems (Minneapolis, MN). Tumor tissues were excised and homogenized in cell lysis buffer (10 mM HEPES (pH 7.9), 1 mM EDTA, 60 mM KCl, 0.2% NP-40, 1 mM DTT, 0.5 μg/ml each proteolytic enzymes aprotinin, leupeptin, and pepstatin A). Supernatants were isolated, and samples containing 40 μg of protein, determined by Bio-Rad protein assay (Bio-Rad Laboratories, Hercules, CA), were analyzed for cytokine expression. A minimum of four mice was used for each test group.

In Vivo Tumor Model. Female C3H/HeJCr mice were obtained from the Frederick Cancer Research Center, Frederick, MD. Mice were housed in barrier cages in a light- and temperature-controlled clean room and fed food and water ad libitum. Eight-week-old mice were injected s.c., near the right scapula, with 3 × 106 RIF-1 tumor cells suspended in 30 μl of culture medium as described previously. When the tumors reached ~5-mm diameter, the tumor and peritumoral area were depleted with Nair (Carter-Wallace, Inc., New York, NY). Treatment protocols, described below, were initiated within the next 1–2 days. All experimental protocols were approved by the Roswell Park Institute Animal Care and Use Committee.

In Vitro Treatment of RIF-1 Tumor Cells with Recombinant TNF-α or DMXAA. RIF-1 tumor cells were plated in 96-well plates (1.25 × 104 cells/well). After 24 h incubation, the cells were exposed to various concentrations (0–500 ng/ml) of anti-TNF-α (R&D Systems). In a separate 96-well plate, RIF-1 cells were exposed to various concentrations (0–100 ng/ml) of DMXAA. After 48 h, the viability of the tumor cells was evaluated by adding 10 μl of a 4 mg/ml 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide solution to each well and reading the absorbance at 560 nm after 4 h of incubation.

Photodynamic Therapy of Tumors. Tumor-bearing C3H/HeJCr mice were injected i.v. (tail vein) with graded doses of Photofrin in a volume of 0.01 ml/g body weight. Approximately 24 h later, the unanesthetized mice were restrained in Plexiglas holders designed to allow exposure of the RIF-1 tumors on the right scapula, with 3-mm diameter, the tumor volume was measured every 1–3 days for up to 90 days; mice were humanely killed when tumors exceeded a volume of 400 mm3.

Analysis of Blood Vessel Density. Tumor including surrounding normal tissue was fixed in zinc fixative and embedded in paraffin according to standard procedures (19). Tissues were sectioned at 5-μm thickness, mounted on positively charged slides, and stained with anti-mouse CD31 monoclonal antibody (PharMinGen). Vessel count was determined by counting the stained vessels in a field of 0.76 mm2 at a magnification of ×200. Ten fields per histological section were included in the analysis. The statistical differences for vessel counts were calculated using Dunn’s post-test after the Kruskal-Wallis test (GraphPad InStat version 3.05 for Microsoft Windows; GraphPad Software Inc., San Diego, CA).

Measurement of Tumor Vascular Perfusion. Tumor perfusion after PDT and DMXAA monotherapies, and DMXAA and PDT in combination, was examined by the measurement of fluorescein fluorescence intensity as described previously (20). For DMXAA alone, RIF-1-bearing mice were given 20 mg/kg DMXAA i.p. and then injected i.v. (retro-orbital route) with 20 mg/kg fluorescein either 1, 2, 3, or 24 h later. For PDT alone, mice were injected i.v. (tail vein) with 1.5 mg/kg Photofrin followed approximately 24 h later by local exposure of the RIF-1 tumors to 135 J cm−2 630-nm laser light. Fluorescein was then administered either 0.1, 1, 2, 3, or 24 h later. For DMXAA plus PDT, mice were treated with PDT as above, except that 20 mg/kg DMXAA was injected i.p. 2 h before local illumination of the RIF-1 tumor. Fluorescein was administered 1, 3, 8, or 24 h later. All of the mice were euthanized 2 min after fluorescein injection, and the tumors were removed and dissolved overnight in Solvable tissue solubilizer, as described previously. Fluorescein fluorescence emission spectra (λex, 460 nm) were collected on a spectrofluorometer (model FP777; JASCO, Tokyo, Japan). The tumor fluorescein uptake was calculated by comparing the fluorescein fluorescence intensity for each tissue sample to a standard curve constructed from dissolved tumor tissue to which known concentrations of fluorescein were added. This method achieves identical results to tumor perfusion measurements by 15RbCl uptake (21) but avoids the handling of radioactive material. Data from at least three mice per time point per experiment, from two different experiments, were analyzed using either unpaired t tests or Mann-Whitney tests (GraphPad InStat version 3.05 for Microsoft Windows; GraphPad Software Inc.).

Combination Therapy and the Effect of Anti-rMuTNF-α Antibody on Tumor Response. In all of the studies, RIF-1 tumor-bearing C3H/HeJCr mice were injected with Photofrin and exposed to 630-nm laser light ~24 h later, as described above. In the first study, the importance of sequence and interval between the application of DMXAA and PDT was examined. For this, 20 mg/kg DMXAA was injected at various times (0.1, 1, 2, 3, and 24 h before the start of illumination, or 1, 2, 3, and 24 h at the end of illumination) relative to the application of the Photofrin-activating light. In a second study, 20 mg/kg DMXAA was injected 2 h before the application of Photofrin-activating light, and the Photofrin dose was varied (1, 1.5, 2, or 4 mg/kg). In a third study, the effect of the neutralizing anti-TNF-α monoclonal antibody (anti-rMuTNF-α antibody) on combination therapy was studied. For this, 50–100 μg of anti-rMuTNF-α antibody was diluted in D5W and injected i.p. in a volume of 0.2 ml for 2 h before the injection of 20 mg/kg DMXAA; DMXAA was injected 2 h before the application of Photofrin-activating light, where the Photofrin dose was either 1.5 or 4.0 mg/kg. To determine whether anti-rMuTNF-α antibody had an effect on PDT alone, mice were injected with 4 mg/kg Photofrin 24 h before light application and then with 50 μg of antibody 4 h before light application. For all of these experiments, the tumors were measured every 1–3 days for up to 90 days; mice were humanely killed when tumors exceeded volume of 400 mm3.

Tumor Response and Analysis. Tumor diameters were measured with vernier callipers and volume was calculated from the formula 0.52L × W2, where L is the longest tumor axis. The hours-to-event, i.e., the time to 400 mm3 tumor volume, was calculated from the monotherapy mouse by interpolating between the times just before and after this volume was reached, using log(tumor volume) for the calculations; both tumor volume and hours-to-event calculations were performed using Excel (Microsoft, Redmond, WA). The median time to 400-mm3 tumor volume, along with the 95% confidence interval for the median, was estimated using the technique of Kaplan and Meier (22), with the Prism (version 3.02; GraphPad Software Inc.) and SAS (version 8.2; SAS Institute, Cary, NC) statistical software packages. Selected event curves were compared by Prism and SAS, using the log-rank test, which calculated a two-tailed P testing the null hypothesis that the curves were identical. Simple linear models were fit to the Photofrin dose-response median times to 400 mm3.
tumor volume, with nonweighted linear regression with SigmaPlot (SPSS, Chicago, IL).

**Normal Tissue Treatment and Response.** Non-tumor-bearing C3H/HeJ mice were restrained in Plexiglas holders designed to expose only the right hind foot to laser light. For these combination studies, Photofrin was injected i.v. at a dose of either 1.5 or 4 mg kg\(^{-1}\) ~ 24 h before applying the laser light whereas DMXAA was administered i.p. at a dose of 20 mg kg\(^{-1}\) ~ 2 h before light exposure. Each treated foot was always compared with the contralateral hind foot. The feet were observed daily and graded according to the following scale: 0, no reaction; 0.1, very slight edema; 0.2, slight erythema; 0.3, slight edema; 0.4, slight edema + slight erythema; 0.5, moderate edema; 0.6, moderate edema + slight erythema; 0.7, large edema; 0.8, moderate erythema; 1.0, erythema + edema and/or slight epilation; 1.1, large edema + erythema/slight epilation; 1.2, large erythema + slight epilation and/or edema; and 1.3, moderate epilation and/or moderate edema. This grading system is subjective and non-linear with dose. Normal tissue response was compared between groups with the Mann-Whitney test.

**Results**

**Enhancement of PDT by DMXAA Was Timing and Sequence Dependent.** The effect of combining DMXAA and PDT was investigated in a transplanted RIF-1 murine tumor model. We first assessed the importance of sequence and interval for the combined treatments. In preliminary experiments the mice were treated with graded doses of either PDT alone (using a fixed light dose but different Photofrin doses) or DMXAA alone, and tumor volumes were measured for up to 90 days (data not shown), as described in “Materials and Methods.” The data were analyzed using the method of Kaplan and Meier, using the estimated median time for tumor (regrowth) to a volume of 400 mm\(^3\) as the end point. The doses of the single agents that were found to be minimally effective in terms of growth delay, 20 mg kg\(^{-1}\) DMXAA and 1.5 mg kg\(^{-1}\) Photofrin (followed ~24 h later by 135 J cm\(^{-2}\) of 630-nm light), were used for the sequence-time interval studies. Neither DMXAA nor PDT at these doses showed any signs of systemic toxicity. As shown in Fig. 1A, tumor regrowth after treatment with PDT alone (median time to 400 mm\(^3\), 12.9 days) was not significantly different from the untreated control growth rate (12.7 days; \(P = 0.10\)), whereas treatment with DMXAA alone resulted in only a slightly longer growth delay (13.2 days; \(P = 0.04\)) compared with controls. The administration of DMXAA 2 h before the PDT light was the most effective combination, achieving a tumor growth delay of an additional ~21 days (median time to 400 mm\(^3\), 34.2 days; 3 of 17 mice with 90-day cures) over untreated or monotherapy-treated tumors. DMXAA administered 2, 3, or 24 h after PDT-light delivery had little effect on tumor response (13.2, 13.1, and 12.9 days, respectively; all \(P_s\) were >0.07 versus control). By contrast, all other intervals resulted in statistically significant delays in tumor regrowth (all \(P_s\) were <0.002). In general, the administration of DMXAA after PDT was ineffective. Overall, the time course pattern in Fig. 1A shows a gradual increase in the efficacy of the combination treatment as the time of DMXAA injection approaches the time of PDT-light treatment, a peak of efficacy at 2 h before light, and then a dramatic exponential decrease of efficacy.

**DMXAA Modified the PDT Antitumor Response by a Factor of 2.81.** For these studies, DMXAA dose and PDT-activating light dose were held constant at 20 mg kg\(^{-1}\) and 135 J cm\(^{-2}\), respectively, whereas the Photofrin dose was varied. Delays in tumor regrowth increased with Photofrin dose, where administration of DMXAA 2 h before PDT with 4 mg kg\(^{-1}\) Photofrin resulted in 5 of 5 = 100% 90-day cures, versus 4 of 11 = 36% 90-day cures for PDT alone. For comparison, a PDT-monotherapy dose of 8.0 mg kg\(^{-1}\) Photofrin is required to obtain 100% 90-day cures of RIF-1 tumors (data not shown). The ratio of the slopes of the regression lines in Fig. 1B gave a DMF of ~2.81.

Normal Tissue Phototoxicity after DMXAA Enhancement of PDT Appeared to be Less Than Additive. The effect of DMXAA plus PDT, using 1.5 or 4.0 mg kg\(^{-1}\) Photofrin, on normal mouse tissue was determined using a foot-response assay. As shown in Fig. 2, there was little difference between the responses obtained when PDT, using 1.5 mg kg\(^{-1}\), was combined with 20 mg kg\(^{-1}\) DMXAA and when PDT was given alone (maximum foot-response score, 0.22 ± 0.05 versus 0.18 ± 0.05, respectively; \(P = 0.58\)). As expected, the normal tissue phototoxic response was greater when 4 mg kg\(^{-1}\) Photofrin was used. And, at this photosensitizer dose, the addition of DMXAA resulted in a statistically significant increase in toxicity (maximum foot-response score, 1.25 versus 0.6 for DMXAA plus PDT versus PDT only, respectively; \(P = 0.0001\)). However, this combination-therapy response was markedly less than that after PDT
monotherapy (foot-response score, 2.46 ± 0.02) using a Photofrin dose (8 mg kg⁻¹) that yields 100% 90-day tumor cures.

DMXAA but not PDT Induced TNF-α in RIF-1 Tumors. The induction of TNF-α by DMXAA and PDT monotherapies and combination therapies, as a function of time after DMXAA injection (or an equivalent time in the case of PDT monotherapies where no DMXAA was given), was measured by ELISA. As shown in Fig. 3A, minimal TNF-α protein expression was observed in control tumors or tumors treated with PDT using either 1.5 mg kg⁻¹ or 4 mg kg⁻¹ Photofrin. Conversely, TNF-α concentrations increased strikingly within 2 h of DMXAA injection. The levels decreased thereafter but remained above the control level for up to 6 h. Interestingly, for the combination therapies TNF-α induction, measured 4.5 h after DMXAA administration, was markedly reduced relative to that obtained after DMXAA monotherapy and in proportion to the subsequent PDT dose, from 50.6 pg of TNF-α (per 40 µg of total protein) recovered 4.5 h after DMXAA alone to 30.1 pg of TNF-α (P = 0.13 versus DMXAA alone) after DMXAA plus PDT with 1.5 mg kg⁻¹ Photofrin and to 17.2 pg of TNF-α (P = 0.016 versus DMXAA alone) after DMXAA plus PDT with 4.0 mg kg⁻¹ Photofrin.

TNF-α and DMXAA Were Not Toxic to Cultured RIF-1 Cells. In vitro assessment of the sensitivity to TNF-α showed that RIF-1 cells were mostly resistant, maintaining a viability ≥74% after exposure for 48 h to rMuTNF-α (0.5–500 ng ml⁻¹). Similarly, RIF-1 cells were resistant to DMXAA with viabilities ≥96% after exposure for 48 h to up to 100 µg ml⁻¹; survival decreased to 55% after incubation with 1 ng ml⁻¹ DMXAA (data not shown).

Neutralizing Anti-Mouse TNF-α Monoclonal Antibody Eliminated the Enhancement of Low-Dose but not High-Dose PDT Antitumor Response. For these experiments, DMXAA and PDT were combined using the schedule that produced the maximum tumor response in Fig. 1A (1.5 mg kg⁻¹ Photofrin; DMXAA 2 h before 135 J cm⁻² light). An i.p. injection of 50 µg of anti-rMuTNF-α antibody resulted in a significant reduction in the median time for tumor regrowth (Fig. 3B) from 34.2 days for the combination therapy alone to 13.6 days for the combination therapy plus antibody (P < 0.0001); the tumor response to combination therapy plus antibody was not significantly different from responses to either PDT or DMXAA monotherapies (both P = 0.8). However, anti-rMuTNF-α antibody (100 µg) had no effect on tumor response following DMXAA plus PDT with 4 mg kg⁻¹ Photofrin (not shown).

DMXAA plus PDT Caused Tumor Tissue Necrosis and Marked Reduction in Vessel Density and Blood Flow. Histological and immunohistochemical analyses revealed the effect of combined DMXAA and PDT on s.c. implanted tumors and their surrounding tissues. The control RIF-1 tumors were composed of fascicles of bland spindled cells with minimal spontaneous tumor necrosis or hemorrhage (Fig. 4A). The tumors were well vascularized, as seen with the CD31 immunostain. A brisk host mononuclear inflammatory cell infiltrate was present throughout the control tumors. After combined
therapy, the most significant histological differences were observed in tumors excised 24 h after light delivery (tumors were excised either 2.5, 5.5, or 24 h after PDT). Both the PDT-alone and DMXAA-alone treated tumors exhibited scattered geographic zones of tumor necrosis and hemorrhage with excellent preservation of intratumoral CD31 immunoreactive endothelial cell-lined blood vessels compared with controls. The combination-treated tumors exhibited extensive tumor necrosis with prominent hemorrhage and virtual absence of tumor vascular CD31 immunoreactivity. Ghost outlines of RBC-filled vascular channels associated with fibrin deposition were identified throughout these tumors. Notably, there was preservation of CD31 immunoreactivity in the peritumoral blood vessels in the combination group that was similar to that seen in either the control or PDT-alone or DMXAA-alone treated tumors. CD31 counts are summarized in Table 1, where a significant (P < 0.001 versus controls, PDT-alone or DMXAA-alone) reduction in CD31 immunoreactivity in the RIF-1 receiving the combination therapy alone was obtained.

Because high doses of PDT and DMXAA reduce tumor blood flow, we also evaluated tumor tissue perfusion using a fluorescein angiography method modified for PDT studies (20). Under our low-dose conditions, neither of the single agents (20 mg kg⁻¹ DMXAA or 1.5 mg kg⁻¹ Photofrin plus light) induced significant changes in blood flow. However, a rapid and marked reduction of blood flow (>70% inhibition at 24 h; P < 0.01 versus control) in the tumors was observed after combined treatment (Fig. 4B).

Discussion

In this study we have demonstrated a large, schedule-dependent enhancement of PDT by the antitumor agent DMXAA. Tumor cures in experimental models and humans can be obtained with PDT, but this can be at the expense of undesirable local or systemic phototoxicity. Likewise, DMXAA is an effective antitumor treatment in murine systems, but significant cures are obtained only at doses approaching the maximum tolerated dose. We have shown previously that the experimental antitumor activity of PDT, but not normal tissue phototoxicity, is greatly enhanced by exogenous TNF-α (4). Because the antitumor effects of DMXAA are, to a great part, mediated through the local induction of TNF-α, we hypothesized that DMXAA would improve both the efficacy and selectivity of PDT. To test this hypothesis, we initially chose doses of DMXAA and PDT that were not sufficient as single agents to inhibit tumor growth. The data show that the combined treatment with low-dose (20 mg kg⁻¹) DMXAA and low-dose (1.5 mg kg⁻¹ Photofrin plus light) PDT led to a reduction in RIF-1 tumor volume with a long delay in regrowth and resulted in an 18% cure rate at 90 days. This represents an enormous increase in efficacy of PDT in this tumor model. In addition, there was no

Table 1 CD31-positive vessels in RIF-1 tumors after the combination therapy of PDT with DMXAA

<table>
<thead>
<tr>
<th>Condition</th>
<th>Median CD31 counts/tumor</th>
<th>P*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>47</td>
<td></td>
</tr>
<tr>
<td>PDT alone</td>
<td>40</td>
<td></td>
</tr>
<tr>
<td>2.5 h</td>
<td>35</td>
<td></td>
</tr>
<tr>
<td>24 h</td>
<td>34</td>
<td></td>
</tr>
<tr>
<td>DMXAA alone</td>
<td>38</td>
<td></td>
</tr>
<tr>
<td>2.5 h</td>
<td>28</td>
<td></td>
</tr>
<tr>
<td>5.5 h</td>
<td>28</td>
<td></td>
</tr>
<tr>
<td>24 h</td>
<td>30</td>
<td></td>
</tr>
<tr>
<td>DMXAA + PDT</td>
<td>34</td>
<td></td>
</tr>
<tr>
<td>2.5 h</td>
<td>28</td>
<td></td>
</tr>
<tr>
<td>5.5 h</td>
<td>28</td>
<td></td>
</tr>
<tr>
<td>24 h</td>
<td>6</td>
<td></td>
</tr>
</tbody>
</table>

*Control, intraperitoneal saline injection only; PDT alone, 1.5 mg kg⁻¹ Photofrin i.v. followed 24 h later by 135 J cm⁻² 630-nm laser light; DMXAA alone, 20 mg kg⁻¹ i.p. DMXAA; DMXAA + PDT, same as PDT alone, but with DMXAA injected 2 h before laser light.

A total of 10 different 0.76 mm² fields were counted/tumor at a magnification of X200, two to three mice per treatment condition.

**P*s were determined with the Dunn’s post-test after a Kruskal-Wallis test.

RIF-1 tumors were excised and fixed 2.5, 5.5, or 24 h after injection of either saline (control and PDT-alone groups) or DMXAA (DMXAA-alone and DMXAA + PDT groups).
that treatment with PDT decreased the amount of measurable TNF-α and time interval. When DMXAA was administered 1–3 h before PDT-activating light, the antitumor activity appeared to be larger than a simple additive effect of each agent; the 2-h interval was particularly effective, yielding a median delay in regrowth of the RIF-1 tumors of over 1 month. Conversely, there was almost no delay in tumor growth when PDT preceded DMXAA. DMXAA administration either 24 h before or immediately before PDT resulted in an intermediate enhancement of tumor response. The reason for this dependence on schedule is not clear. Because both PDT and DMXAA are antivascular agents, we questioned whether the very low antitumor activities of some of the therapeutic schedules were attributable to reductions in blood flow sufficient to impair delivery of either DMXAA (when PDT preceded DMXAA) or oxygen (when DMXAA preceded PDT); the effectiveness of PDT depends on the presence or absence of molecular oxygen) to the tumor (2). However, this explanation appears unlikely because vascular perfusion, as measured by fluorescein fluorescence, did not decrease markedly after either of the low-dose agents. Our anti-TNF-α antibody experiments suggest that the enhancement of PDT by DMXAA critically involves induction of TNF-α (at least for the regimen where DMXAA was administered with low-dose PDT). However, the ELISA data in Fig. 3A indicate that treatment with PDT decreased the amount of measurable TNF-α induced by DMXAA in the RIF-1 tumor. It is possible, therefore, that the lack of antitumor effect seen when DMXAA was administered after PDT was a result of a similar reduction. Photofrin-PDT has been shown previously to induce TNF-α and other inflammatory cytokines after the treatment of urinary bladder cancer in human patients (25), although neither low-dose nor high-dose Photofrin-PDT led to the induction of TNF-α in our study. Other reports demonstrate either the presence (26, 27) or absence (27) of TNF-α induction in tumor-bearing animals after PDT using different photodynamic sensitizers. A potential, but not yet confirmed, explanation for the lack of interaction when PDT is given before DMXAA is the recent observation that PDT-treated normal and malignant cells can immediately, but temporarily, lose their responsiveness to some inflammatory cytokines and growth factors via rapid receptor loss (28).

In the previous report of Bellnier (4), the addition of exogenous TNF-α enhanced the activity of PDT against implanted SMT-F tumors but showed little potentiation of normal tissue phototoxicity. In the present study, there was an analogous lack of normal foot-tissue phototoxicity after combined DMXAA and low-dose PDT. Our immunohistochemical analysis of RIF-1 tumors and surrounding cutaneous and s.c. tissue revealed similar sparing of the normal tissue in the tumor treatment field. Exposure of mouse feet to a PDT-therapy dose (8 mg kg⁻¹ Photofrin) that also yielded 100% 90-day tumor cures led to severe damage (see Fig. 2). However, when we combined DMXAA and one-half that PDT dose, we obtained an identical tumor cure rate but markedly less normal tissue damage. Together, these data indicate a therapeutic advantage to our approach to enhancing PDT with low-dose DMXAA.

Because the goal of this study was to enhance PDT with an agent that induced TNF-α locally in tumors, we only focused on a dose of DMXAA that showed little antitumor activity. Preclinical studies have shown that doses of DMXAA higher than those used here selectively and irreversibly inhibit tumor blood flow, resulting in hemorrhagic necrosis. Notably, DMXAA appears to be more effective in causing necrosis to the poorly perfused central region than to the periphery of tumors. Vascular-targeted therapy of cancer is highly attractive because it has the potential to overcome problems associated with therapies that target tumor cells themselves, such as tumor heterogeneity and drug resistance. However, drugs that target tumor vascular endothelium have met with limited success as single agents, often failing to eliminate viable cells on the periphery of the tumor. Strategies that combine the targeting of established tumor vasculature, including the administration of DMXAA, with standard anticancer therapies have been developed recently (see Ref. 15 and citations therein). We have begun studies using larger doses of DMXAA that provide antitumor activity, in combination with both Photofrin-PDT and ALA(protoporphyrin-IX)-PDT, with the hopes of improving the therapeutic synergism reported here. Effective monotherapeutic doses of DMXAA and PDT should have complementary activity, with DMXAA being more active against poorly vascularized areas of the tumor and PDT more active against well-oxygenated regions. ALA-PDT is more directly toxic toward tumor cells than conventional Photofrin-PDT and can be applied clinically in a way as to not induce cutaneous photosensitivity. Therefore, the combination of DMXAA with ALA-PDT should be able to exploit further their different cellular targets and non-overlapping toxicities.

Acknowledgments

We thank Patricia Maier for technical assistance and Drs. Barbara W. Henderson and Thomas J. Dougherty for helpful discussions.

References


Treatment with the Tumor Necrosis Factor-α-Inducing Drug 5,6-Dimethylxanthenone-4-Acetic Acid Enhances the Antitumor Activity of the Photodynamic Therapy of RIF-1 Mouse Tumors

David A. Bellnier, Sandra O. Gollnick, Susan H. Camacho, et al.


Updated version
Access the most recent version of this article at:
http://cancerres.aacrjournals.org/content/63/22/7584

Cited articles
This article cites 23 articles, 7 of which you can access for free at:
http://cancerres.aacrjournals.org/content/63/22/7584.full#ref-list-1

Citing articles
This article has been cited by 2 HighWire-hosted articles. Access the articles at:
http://cancerres.aacrjournals.org/content/63/22/7584.full#related-urls

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.