Novel Photodynamic Effects of a Benzophenothiazine on Two Different Murine Sarcomas

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ABSTRACT

The photochemotherapeutic properties of a novel benzophenothiazine, 5-ethylamino-9-diethylaminobenzo[a]phenothiazinium chloride, were assessed in vitro and in vivo against two murine mammary sarcoma models (EMT-6 and RIF). Photodynamic therapy (PDT) of EMT-6 and RIF cells following a 30-min incubation with dye (0.4 μg/ml) and a light dose of 3.3 J/cm² killed 87.0 and 99.6% of the cells, respectively. Over this same time period, RIF cells accumulate more than twice the amount of dye than the EMT-6 cell line (7.54 ± 0.17 (SD) versus 3.11 ± 0.15 nmol/10⁶ cells) which probably accounts for their increased sensitivity to PDT. Conversely, in vivo, the EMT-6 tumor accumulates 3 times more dye (34.66 ± 2.16 μg/g dry weight) than the RIF tumor (12.28 ± 1.27 μg/g dye)/3 h post-s.c. injection of dye (15 mg/kg). A study of the concentration dependent uptake of dye (following s.c. injection) in the tumor and plasma of mice bearing the EMT-6 tumor indicated a nonlinear relationship for both compartments. Maximum tissue uptake of dye and discrimination between tumor and skin or muscle occur 3–8 h following s.c. injection of dye. The ratios of dye in the tumor to the dye in surrounding skin and gastrocnemius muscle 8 h following dye injection were 4:1 and 8:1, respectively. At 24 h after dye injection, the dye was not detectable by absorption spectroscopy in the tumor, skin, or muscle. Decreasing the fluence rate from 200 to 50 mW/cm² at a total light dose of 100 J/cm² optimized the PDT effect. At 3 h following s.c. administration of dye, PDT of EMT-6 (7.5 mg of dye/kg; 50 mW/cm²; 100 J/cm²) and RIF tumors (15 mg dye/kg; 50 mW/cm²; 150 J/cm²) resulted in 100 and 70% cures, respectively. Histology at 24 and 72 h post-PDT showed minimal or no damage to the surrounding tissue (skin) while 70–90% of the tumor cells were destroyed or damaged. Moreover, 50–60% of the tumor cells isolated and cultured immediately following PDT were found to be nonviable. Similarly, the administration of 60 mg 5-ethylamino-9-diethylaminobenzo[a]phenothiazinium chloride/kg also resulted in no damage to the skin 24 h following PDT. It is suggested that the redox properties of the dye coupled with the differing metabolic states of the tumor and skin, which increase the amount of photoactive, oxidized dye present in the tumor and decrease it in the skin, are responsible for this unique differential PDT effect. Histological and fluorescein dye exclusion data 24 and 72 h post-PDT indicated that there is minimal damage to the irradiated vasculature within and surrounding the tumor under conditions which lead to high cure rates. This correlates with the predominant intracellular tumor localization of the dye, demonstrated by fluorescence microscopy, and the low levels of dye (54 ng/ml plasma) found in the plasma at the time of light exposure. Taken together, these results indicate a novel PDT effect in that direct tumor cell killing (without skin photosensitization) rather than destruction of the supporting vasculature is the primary mode of tumor irradiation.

INTRODUCTION

PDT is a promising new approach for the selective eradication of neoplastic tissue which does not result in the deleterious side effects that are often experienced with both chemotherapy and ionizing radiation therapy (see reviews in Refs. 1–6). PDT involves the systemic administration of tumor localizing photosensitizers that can inactivate malignant tissue when irradiated with light of the appropriate wavelength. Photoactivation of the photosensitizer in the presence of oxygen generates highly reactive and cytotoxic molecular species (7–10) by one or both of the following mechanisms: (a) a type I reaction where the excited state of the dye interacts directly with biomolecules to generate free radicals, hydrogen peroxide, superoxide, etc.; or (b) a type II reaction where the excited dye interacts directly with oxygen to generate the highly reactive, short-lived cytotoxic singlet oxygen (¹O₂). In vitro these oxidizing species cause cell death as a result of damage to various cellular organelles and functions depending on the photosensitizer used. In vivo, however, studies of the acute effects of PDT on animal tumors using a variety of sensitizers have shown that vascular occlusion is largely responsible for tumor eradication (11). This treatment modality has progressed to phase III clinical trials using two PDT drugs, HPD and PII. Although encouraging results have been obtained with these PDT agents for a wide variety of neoplasms, it has become apparent that in order to realize the full potential of PDT additional sensitizers must be developed. The reported limitations (2, 4) of both HPD and PII include: (a) a low absorption coefficient in the region where activating light penetrates tissue most efficiently (600–900 nm); (b) both products are not a single entity but consist of mixtures of porphyrin ether and ester oligomers; (c) prolonged retention in the skin leads to dermal photosensitization that can persist for months; and finally (d) the rapid formation of hypoxic cells which occurs as a consequence of the vasculature damage during PDT with these photosensitizers increases the probability that a fraction of the tumor cells will escape direct photodestruction (11, 12). Nutritional resupply to these still viable tumor cells through diffusion or angiogenesis may rapidly repopulate the tumor (6).

The successes of PDT and the limitations of HPD and PII have stimulated the search for more efficacious phototoxic compounds primarily within the porphyrin family (i.e., benzoporphyrins, chlorins, purpurins, phthalocyanines, bacterio-chlorophyll-a, etc.); thus these second generation drugs tend to have similar PDT properties (4). Our approach for extending the applicability of PDT is to study diverse classes of photosensitizers which possess intrinsically different physicochemical and pharmacological properties. A potential novel class of photosensitizers for PDT came to our attention as a result of the investigations of Lewis et al. (13, 14) into the antitumor activity of the benzophenoxazine family of cationic dyes. Their investigations demonstrated the propensity of benzophenoxazines to selectively stain and inhibit the growth of tumors (transplanted murine mast cell sarcoma and a spontaneous adenocarcinoma). This finding was substantiated by the work of Bates and Kerschmann (15) who also observed selective staining of a variety of transplanted and induced murine brain tumors using a member of this family of dyes. This prompted us to investigate these dyes as PDT agents. Unfortunately, the compounds studied by Lewis were found to be photoinactive (16). We therefore designed and synthesized a large number of novel drugs belonging to this family of chromophores which possess increased photocytotoxic properties (16–19). We have shown that a correlation exists between the in vitro photocytotoxicity of the benzophenoxazine derivatives and their
ability to generate *O₂*, although contributions from a Type I mechanism cannot be ruled out (16, 19, 20). We have also systematically evaluated structure/function relationships with respect to the uptake, retention, localization, and phototoxicity of these dyes in a variety of cancer cell lines (16–22). These *in vitro* investigations have established the fact that this family of dyes possess not only the fundamental characteristics necessary for a photochemotherapeutic agent, high photocytotoxicity with a concomitant low cytotoxicity, but also other attributes which could be beneficial for the treatment of malignant and infectious disorders. These include: (a) a high degree of lipophilicity; (b) rapid intracellular accumulation; and (c) efficient absorption of light in a spectral region where light penetrates tissue maximally. In addition, our preliminary *in vivo* studies indicate that, unlike the porphyrin derivatives, the benzophenoxazine analogues seem to rapidly accumulate intracellularly and cause tumor destruction with minimal damage to the vasculature (18).

The primary objective of the present *in vivo* study is to ascertain whether the photocytotoxic efficacy observed *in vitro* with these novel dyes may be extended to animal model systems and whether the mode of tumor eradication involves substantial initial damage to the vasculature as with the porphyrin derivatives. As such, an attempt is made to optimize the physical parameters of dosimetry (power and energy density) delivered to the tumors. We have chosen to examine the phototoxic behavior of EtNBS for several reasons: (a) it is photocytotoxic (18, 20) to a variety of cancer cell lines; (b) the dye is easily prepared as a pure single material; and (c) its solubility in aqueous media simplifies its administration to animals. The mode of tumor eradication was assayed by histological analysis, fluorescein dye exclusion, and the inhibition of prostaglandin synthesis with indomethacin.

### MATERIALS AND METHODS

**Chemicals.** EtNBA and EtNBS were prepared by established procedures described in US Patent 4,962,197 and by Clapp et al. (23) and Crossley et al. (24). Their specific synthesis will be reported separately. All dyes were purified by medium pressure (100 psi) liquid chromatography. Silica gel (Woelm 32-63) was used as the solid phase, eluting with a linear gradient of methylene chloride:methanol (100:0-90:10). They were homogeneous by thin layer chromatography and high field nuclear magnetic resonance spectroscopy (JEOL 63 MHz).

**Cell Culture.** The EMT-6 tumor cell line, a murine mammary carcinoma syngeneic to BALB/c mice, was obtained from Dr. E. Lord of the University of Rochester Cancer Center, Rochester, NY, and maintained according to the protocol of Rockwell et al. (25). Radiobiological and immunological characteristics of this cell line have been described previously (26). The cells were cultured in RPMI 1640 (Sigma Chemical Co., St. Louis, MO) supplemented with 1% fetal bovine serum from Sigma, 10 units/liter penicillin, 100 mg/liter streptomycin, 25 mm sodium bicarbonate, and 3.5 µl/liter of 2-mercaptoethanol (Sigma). RIF tumor cells (passage 5) derived from a radiation-induced fibrosarcoma carried in C3H/HeJ mice were obtained from Dr. Allan Osoroff of the Roswell Park Memorial Institute, Buffalo, NY, and maintained through *in vivo*/*in vitro* passages according to established procedures (27). RIF cells were cultured in complete minimum essential medium α (Sigma) supplemented with 10% fetal bovine serum, 0.75 ml/500 ml of 200 mm l-glutamine, and antibiotics as above. Both cell lines were incubated at 37°C in a humidified 95% air:5% CO₂ atmosphere.

**In Vitro Dye Uptake Studies.** Subconfluent cell cultures in the log phase of growth were utilized for dye uptake studies at approximately 3 × 10⁶ cells/25-cm² flask (*n* = 6). Medium was aspirated from cultures and replaced with 2 ml of phenol red free HBSS containing 3 µM dye. Cells were then incubated in the dark at 37°C for 30 min, a time which had been previously shown to result in maximum dye uptake for a wide range of extracellular dye concentrations (20). Cells grown in culture flasks were washed twice with HBSS, trypsinized, placed in Eppendorf tubes, and pelleted by centrifugation (8000 × g). Benzophenothiazine was extracted from the resultant pellet with 0.7 ml of methanol:chloroform (1:1) containing 5 µl of acetic acid/ml (19). The amount of dye present was then quantitated spectrophotometrically (Perkin-Elmer Lambda 5 UV/VIS). Prior to centrifugation an aliquot was taken for the determination of total protein by a modified Lowry method using a commercially available kit (Sigma No. 690 A). All manipulations of cells exposed to photosensitizer were performed in subdued light. For the sake of comparisons it should be noted that 1.0 µM dye corresponds to 0.4 µg dye/ml.

**In Vivo Phototoxicity Studies.** For comparative purposes the photocytotoxicity of EtNBS toward the RIF cell line was determined under the same conditions as previously used for EMT-6 cells (20). An appropriate number of cells (4 × 10⁶/0.5 ml) were seeded in a 24-well, flat bottomed Linbro plate (2 cm²/well). Forty-eight h later, subconfluent monolayers were incubated for 30 min in the dark with 1 ml HBSS containing either 1.0 µM sensitizer or with HBSS alone at 37°C. Following incubation, all cultures within each group (dye or HBSS) were washed twice with HBSS and either subjected to light exposure (10 min; 3.3 J/cm²; 590-700 nm) or maintained in the dark in HBSS at 20°C. After treatment all cultures were placed back into growth media at 37°C until assayed for cell viability. The light source was a Polaroid 610 slide projector and is described in detail elsewhere (16). Cell viability was determined by 0.4829 following light exposure via [³H]thymidine incorporation (ICN Radiochemicals, Irvine, CA) into cellular DNA. Cells were exposed to [³H]thymidine (1 µCi/0.5 ml; specific activity, 50 Ci/mmol) in supplemented MEM for 6 h and then washed three times with HBSS and three times with 5% trichloroacetic acid to precipitate the DNA. Cellular precipitates were dissolved in 0.5 N sodium hydroxide and counted for ³H content. The percentage of viable cells following light exposure was calculated as

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\text{dpm of cells + dye + light} \\times 100
\]

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\text{dpm of cells + dye + dark}
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Similarly, the dark toxicity of the dyes was calculated as

\[
\text{dpm of cells + dye + dark} \\times 100
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\[
\text{dpm of cells + dark}
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Statistical differences in percentage of cell viability between groups were determined by unpaired Student’s *t* test. Freshney (28) points out that [³H]-nucleotide incorporation is a reliable indicator of viability if an appropriate recovery period (several cell population doubling times) is allowed following PDT prior to incorporation measurements. The EMT-6 and RIF cell lines have a doubling time of ~10–12 h. A study of benzophenoxazine cytotoxicity using the standard clonogenic assay was found to be consistent with our [³H]-thymidine uptake results (19). Prior to quantitatively evaluating phototoxic effects the cells were observed with light microscopy. Typically, cell cultures that had sustained serious photochemical damage were found to have severely depleted populations; most remaining cells presented distorted morphologies including cytoplasmic blebbing and vacuolization.

**Tumor Systems.** Male BALB/c and C3H/HeJ mice (21 days old) were obtained from Charles River Breeding Laboratories, Wilmington, MA. The mice were acclimated to the 12:12 light:dark cycle of the animal care facility for at least 1 week prior to inoculation with tumor cells and weighed 20–25 g at the time of PDT treatment. After depilation, mice were inoculated in the right flank with 0.5 × 10⁶ RIF or 1.7 × 10⁶ EMT-6 cells/0.1 ml of media. Mice were used for PDT treatment or dye uptake studies 7–10 days following inoculation when the tumor surface diameter reached 5–7 mm and a thickness of 2–4 mm.

**Tissue Uptake/Retention of Dye.** The retention characteristics of EtNBS in EMT-6 tumor, normal skin, and muscle were evaluated at 1, 3, 8, and 24 h after a s.c. injection of dye (15.0 mg/kg body weight in 0.6 ml isotonic sucrose) in order to determine an optimum time for photoradiation of tumors. At these times the mice (*n* = 5) were sacrificed by cervical dislocation and the tumors, adjacent skin (2 cm²), and gastrocnemius muscle were removed by dissection. The dye content of these tissues was quantitated by extraction for 24 h with methanol:chloroform (1:1) containing 5 µl of acetic acid/ml. The samples were centrifuged and the dye content of the supernatant was quantified spectrophotometrically. Intratumoral dye concentration was expressed as µg of dye/g of dry weight of tissue. Dye retention in the RIF tumors was similarly studied at the 3- and 8-h periods following the same protocol. To study the concentration dependent uptake of EtNBS in the EMT-6 tumor at the time of maximal tumor/normal skin discrimination determined above, different groups of mice (*n* = 5–13) were given a s.c. injection of dye at 3.75, 7.50, 15.0, or 30.0 mg/kg body weight in 0.6 ml of isotonic sucrose. Three h following dye administr-
tion, the mice were sacrificed and the dye content of the tumors was quantified as above. For comparisons with wet tissue weights, it was found that approximately 85% of the tumor mass was viable. In order to determine the most efficient route of dye administration with respect to tumor uptake of dye, EMT-6 tumor bearing mice were given an injection (15.0 mg EtNBS/kg) either s.c. under the scruff of the neck (n = 13) to ensure isolation from tumor site, i.p. (n = 6), or i.v. (n = 7) via the lateral tail vein. Three h (determined from the pharmacokinetic study above) after the administration of dye, the mice were sacrificed and the dye content of the tumors was quantified as above.

**In Vivo Dye Uptake in Plasma.** Dye concentration in the plasma 3 h following the administration of EtNBS at 7.5, 15.0, 30.0, or 60.0 mg/kg body weight was assessed in different groups of mice (n = 4–5). The 3-h time point was chosen since this was the time interval between dye administration and exposure of tumor to light in the PDT experiments. Blood was obtained by decapitation and plasma isolated by centrifugation (14,000 × g) in heparinized centrifuge tubes. The plasma in each group of mice was pooled and the dye concentration was quantitated by fluorescence spectroscopy (Fluorolog 2 spectrofluorometer, Spex Industries, Edison, NJ) using a standard fluorescence curve obtained by adding known amounts of EtNBS to a measured volume of plasma (excitation/emission = 630/690 nm). Results are expressed as ng of dye/100 μl of plasma.

**In Vivo Sensitization and Light Exposure.** The effects of both light energy dose and power density on EMT-6 tumor control were studied in mice given a s.c. injection of dye (7.5 mg/kg body weight in isotonic sucrose) and irradiated 3 h later. First, three different groups of mice (n = 10–13) were each given a total dose of 200 J/cm² at a fluence of either 50, 100, or 200 mW/cm². Tumor exposures to light were carried out on immobilized animals without anesthesia using 652 nm light delivered via a 5-W argon ion pumped tunable dye laser (Coherent model Innova 100, Palo Alto, CA) using 4-(dicyanomethylene)-2-methyl-6-(p-dimethylaminostyryl)-4H-pyran (Exilim Chemical Co., Dayton, OH). The output beam from the dye laser was coupled to a 1-mm quartz fiber optic cable. A microfibre was fitted to the end of the fiber optic cable to ensure an even light distribution throughout the treatment field. The wavelength was tuned with a birefringent filter and the power density was adjusted for a spot size of 1.2 cm which encompassed the tumor as well as some normal tissue. In a second protocol, different groups of mice (n = 10–15) were each irradiated at a fluence rate of 50 mW/cm² and either 50, 100, or 200 J/cm². As a control for photothermal effects, PDT with the photoinactive dye EtNBA was assessed in the EMT-6 tumor mouse (n = 5) were given injections of dye (7.5 mg EtNBA/kg) and irradiated 3 h later with a fluence of 50 mW/cm² and a total energy of 100 J/cm². Control mice included groups which received either no dye/no light (n = 13) or dye with no light (n = 12).

**Histology.** Mice were sacrificed by cervical dislocation 24 and 72 h following light treatment and the entire irradiated area including the tumor, surrounding skin, and underlying muscle was removed and fixed in phosphate buffered formalin. The fixed tissue was embedded in paraffin, sectioned at 5-μm intervals, stained with hematoxylin and eosin, and examined with light microscopy.

**RESULTS**

**Physical and Photophysical Properties.** The molecular structure of the dye used in this study along with its absorption in methanol are shown in Fig. 1. The planar chromophore possesses a delocalized positive charge and absorbs red light efficiently (λ_max = 652 nm; ε = 68,600). Its partitioning coefficient (Pc = 580) between 2-octanol and phosphate buffered saline (pH 7.4) indicates a lipophilicity greater than that of many porphyrins (32) and yet the dye is very readily soluble in aqueous solutions. This is apparently the consequence of the 5-ethyl group which seems to inhibit the formation and precipitation of large dye aggregates since an analogue in which this moiety is replaced with a hydrogen atom is much less soluble in aqueous solution.

**In Vitro Studies.** In order to assess possible differences in the in vivo efficacy of EtNBS in the EMT-6 and RIF tumor models, we...
initially investigated the dye uptake and photocytotoxic effects in these same cells. Dye uptake for cells incubated with 2 ml of 3.0 μM dye for 30 min was 2.5-fold greater for RIF than for EMT-6 cells [7.5 ± 0.2 (SD) versus 3.1 ± 0.2 nmol/10⁶ cells, respectively; \( P < 0.01 \)]. Fluorescence spectroscopy using conditions that are detailed elsewhere (20) verified that the dye had accumulated intracellularly. Likewise, in vitro photocytotoxicity (1.0 μM EtNBS) was greater for RIF than for EMT-6 cells (0.4 ± 0.1 versus 13.0 ± 2.8% viable cells, respectively, \( P < 0.01 \)). This may be related to the higher concentration of EtNBS in the RIF cell line. Henderson et al. (29) has similarly shown that in vitro, RIF cells are more photosensitive to HPD than EMT-6 cells although she presents no data on dye uptake.

**In Vivo Studies.** The pharmacokinetics of dye uptake (15.0 mg/kg) in the EMT-6 tumor, normal skin, and gastrocnemius muscle is presented in Fig. 2. There is a rapid initial uptake in all three tissues with a maximal dye concentration (34.7 ± 2.2 μg/g dry weight) occurring in the tumor approximately 3 h after the s.c. administration of dye. The ratio of dye in the tumor to skin increased from 3:1 at the 1-h measurement to 4:1 at 8 h. During this same time period, the ratio of dye in the tumor to muscle increased from 2:1 to 8:1. At 24 h the dye is not detectable by absorption spectroscopy in any of the tissues. Although the data indicate that there is better discrimination between tumor and tissue at 8 h we opted to perform PDT 3 h after dye delivery because there was 25% more dye present in the tumor at this time and preliminary studies indicated that the level of dye in the skin did not cause deleterious effects. A high concentration of dye is observed in the urine of the mice, usually within 1 h of its administration. To minimize the use of animals, we measured the intratumoral dye concentration only at the 3- and 8-h time points for the RIF tumor model. Although the pharmacokinetics appears to be the same for this tumor as with the EMT-6 (both tumor types lost approximately 55% of their dye content between the 3- and 8-h measurements), the amount of dye taken up by them was substantially different. At 3 h post-dye injection the RIF tumor contained 12.3 ± 1.3 μg of dye/g dry weight, which is approximately 33% of the dye content in the EMT-6 tumor at this time point. Tumor (EMT-6) uptake of dye is not linear with dye concentration but instead follows an S-shaped curve (Fig. 3). It appears that the tumor is saturated around the 15.0-mg/kg dose level (34.7 ± 2.2 μg of dye/g dry weight, of tumor). Visual observation of the s.c. injection site after dissection indicates the presence of dye in the surrounding tissues, possibly accounting for the nonlinear dye uptake by the tumor at low dye concentrations (i.e., the dye must first saturate the injection site tissue before entering the circulation).

Because vascular PDT effects have been correlated to the circulating level of photosensitizer (4, 6), we examined the concentration of EtNBS in the blood plasma at the time of light exposure (3 h after injection) at various dye concentrations. Fig. 4 indicates that there is no linear correlation between the concentration of administered dye and the quantity measured in the blood plasma. At the level of photosensitizer required for cures (7.5 mg/kg), there were only 54 ng of EtNBS/ml of plasma.

The route of administration of sensitizer influenced the level of its distribution to the tumor. Injection of dye (15 mg dye/kg) s.c., i.v., or i.p. resulted in values of 34.7 ± 2.2, 26.0 ± 4.7, or 15.3 ± 0.7 μg of dye/g dry weight, of tumor, respectively, 3 h following its administration. Dissection revealed that the peritoneal cavity contained an appreciable amount of adsorbed dye which probably accounts for the lower uptake values.

It was noted during these extraction studies that a small (2–10-nm) blue shift had occurred in the measured absorption maximum of the dye. This most likely indicates that some type of a metabolic de-saturation is occurring to varying degrees in vivo since this phenomenon is known to occur under oxidizing conditions (33) with a similar class of dyes.

**Tumor Response Studies.** The EMT-6 tumor model was chosen to evaluate both the effect of fluence and total energy dose on the
photodynamic efficacy of EtNBS. Fig. 5 indicates that when mice were given 7.5 mg/kg and illuminated with a total light dose of 200 J/cm² power densities of 50–200 mW/cm² gave significant (P < 0.01) tumor responses compared to nonirradiated control mice as assayed by tumor dry weight. Moreover, lowering the fluence from 200 to 50 mW/cm² significantly (P < 0.01) increased the tumor response. Mice (n = 15) with nonpalpable tumors 14 days post-PDT remained tumor free for 90 days. We next examined the effect of light dose (50–200 J/cm²) on PDT efficacy while maintaining the fluence at the optimal 50 mW/cm² (Fig. 6). There is a dose threshold for PDT effects at around 50 J/cm² and a moderate light dose of 100 J/cm² is as effective as 200 J/cm². At 100 J/cm² no measurable tumors were detectable 14 days post-PDT (n = 15). Although PDT on larger tumors (8–10 mm) resulted in a 100% response rate (decreased tumor dry weights compared to controls) under these conditions (50 mW/cm²; 100 J/cm²), the majority of the tumors were palpable on day 14 post-PDT (data not shown). Similarly, a recent study (34) on the relationship between tumor volume and response to HPD-PDT using the RIF animal model has shown that small tumors are completely eradicated while intermediate and large tumors show either partial or no delay in regrowth, respectively. This effect is most likely due to the limited penetration of light in the larger tumor volumes (2, 5).

Because the known antigenic properties of the EMT-6 tumor (27, 29) may result in an immunological contribution to the PDT response and thus possibly obscure the degree of direct phototoxic effects, we used the nonantigenic RIF tumor system (27) to further investigate the PDT efficacy of EtNBS. We doubled the amount of administered dye (15 mg/kg) and used a slightly greater light dose (150 J/cm²) in this treatment because of the lower dye uptake and the significantly diminished transmission of incident light with this tumor model (29). These conditions resulted in no palpable RIF tumors after 14 days (n = 11) and 72% of these animals showed no sign of tumor regrowth after 90 days.

The first effect observed immediately following the exposure of the EMT-6 tumor to light is a bleaching of the bluish tumor and surrounding skin within the light field. This is most likely a result of the photoreduction of the dye which will be discussed below. The locus of light exposure in both models developed moderate edema but no erythema 2–3 h following PDT. There were also signs of hemorrhage within the tumors but it did not appear prominent. The edema persisted for 24–48 h and was usually gone at 72 h. During this same time period eschar formation occurred to varying degrees directly over the tumor. In the following weeks, the scarred tissue healed and the hair regrew to leave no visible sign of PDT in the cured animals. For those animals not cured, tumor regrowth occurred in the following weeks at the edges of the original tumor. Interestingly, both EtNBS (Fig. 5) and EtNBA (data not shown) had an antimitotic effect on the tumors at the 7.5-mg/kg dose in the absence of light. The chemotherapeutic effects of this class of compounds is consistent with the early observations of Lewis et al. (13, 14).

Histological analysis of the tumor and surrounding tissue at 24 and 72 h post-PDT (under optimal PDT conditions) revealed similar results for both tumor models. At 24 h post-PDT, approximately 70–90% of the tumor mass suffered substantial cell damage characterized by nuclear pyknosis, severely vacuolated cells, and large areas of necrosis including amorphous granular debris. Small areas of viable staining tissue as well as independent cells were localized randomly throughout the necrotic regions. These areas sometimes contained preserved vascular structures. In addition, undamaged tumor cells were often detected around the periphery of the tumor mass. Two consequences of PDT on the tumor microcirculation were obvious: (a) the lumen of many intact capillaries became packed with erythrocytes (stasis); and (b) there was only a moderate extravasation of RBC. Surprisingly, the 72-h histological examination (Fig. 7A) indicates only an additional 5–10% decrease in the number of viable cells compared to the 24-h histology. In striking contrast to the prominent destruction of the neoplastic tissue with EtNBS, the overlying skin displayed near normal histology (Fig. 7C). There was little or no hemorrhage of the microvasculature and minimal or no necrosis of the epidermis or dermis (fat cells and panniculus carnosus muscle cells) in spite of the fact that the total area irradiated (tumor and surrounding normal tissue) showed moderate edema 24 h post-PDT.

Tumors and surrounding tissue treated with either isotonic sucrose or the photoinactive analogue, EtNBA, displayed normal architecture consisting of multiple mitotic cells, easily discernible blood vessels, and few necrotic foci 72 h post-PDT (Fig. 7B). Since EtNBA has physical and photophysical properties very similar to those of EtNBS, lacking only the ability to generate ¹O₂ efficiently (20), it serves as a unique control to differentiate photo-induced thermal effects from photodynamic effects. Consequently, the absence of any observable phototoxicity with EtNBA in vivo strongly suggests that the cytotox-
Fig. 7. Photomicrographs of PDT treated EMT-6 tumors and skin. 
a. histology of EtNBS treated (7.5 mg/kg) tumor (50 mW/cm², 100 J/cm² at 652 nm) 72 h post-PDT. H & E, ×300. Note nuclear pyknosis, vacuolization, and extravasation of RBC into stroma. b. 72-h histology of tumor treated with photoinactive analogue EtNBA (7.5 mg/kg) under the same conditions as above showing no damage to tumor. H & E, ×300. c. the undamaged skin overlying the EMT-6 tumor in a shows minimal damage. d. 24-h histology of BALB/c mouse skin following the administration of 60 mg/kg EtNBS and a 3-h interval before PDT (50 mW/cm², 100 J/cm² at 652 nm). This high sensitizer dose results in minimal effects. H & E, ×300. e. fluorescence micrograph section (10 μm; prepared at ~76°C) of EMT-6 tumor 3 h following the administration of EtNBS (15.0 mg/kg) showing intracellular location of dye. ×400. f. fluorescein angiograph (2 mg/ml) of skin flap containing EMT-6 tumor immediately following PDT (conditions as in a). Note no exclusion of dye to the tumor and surrounding tissue. g and h. 24-h post-PDT fluorescein angiograph of bottom and top of skin flap containing EMT-6 tumor (conditions as in a). Note signs of hemorrhage within the tumor. i. photograph of bottom of skin flap (no fluorescein) 24 h post-PDT (conditions as in a). Note that the major blood vessels leading to the tumor appear intact.

Skin Response Studies. Exposure of the hind quarter of mice to light 3 h following the s.c. administration of either 7.5, 15.0, 30.0, or 60.0 mg of dye/kg resulted in no observable effects to the tissue at 24 h post-PDT. There was neither edema nor erythema even at the highest dye dose given (60.0 mg/kg) which resulted in an uptake of 49.9 ± 5.2 μg of dye/g dry weight of skin at the time of light exposure. Histological examination (24 h post-PDT) of skin from mice given this high dye dose showed no or minimal effects (Fig. 7D). However, at this high drug dose all of the mice died 24–48 h after PDT due to some type of unknown mechanism. When mice were given the same dose of dye and maintained in subdued light for 14 days, they showed no visible signs of distress or injury in the absence of PDT. This supports earlier findings which indicate that there is minimal systemic toxicity with this class of dyes (13–15) and that the observed toxicity is induced by PDT. This phenomenon of acute toxicity following PDT in experimental animals has been reported previously when using porphyrin type photosensitizers (2, 31). It has been presumed that the relationship of the PDT treatment area to total body area is an important parameter in the induction of the acute lethality since equivalent doses of drug do not appear to cause an effect in larger animals.
Evidence for Direct Cell Killing following in Vivo PDT. Several pertinent experiments were carried out in order to assess the contribution that vascular damage has on the PDT induced tumor regression. Unfortunately, we were not able to perform a quantitative in vivo/in vitro experiment as described by Henderson and Fingar (11) where cell killing is investigated in cell culture after the in vivo administration of dye because of the relatively rapid efflux of EtNBS from the cells during the isolation protocol. However, an in vitro analysis of EMT-6 tumor cell survival immediately following in vivo PDT (50 mW/cm²; 100 J/cm²) indicates that 50–60% of the cells are directly killed. This most likely indicates that a significant portion of the dye resides in the cancer cells of the tumor. Examination of thin sections of a similar tumor (Fig. 7E) with fluorescence microscopy 3 h following the in vivo administration of dye (15.0 mg/kg) clearly showed dye fluorescence originating from tumor cells. Vascular damage (occlusion) to the irradiated area under PDT conditions which lead to tumor cures with the EMT-6 model was assessed by the fluorescein dye exclusion method. Fluorescein injection immediately after PDT showed no exclusion of dye to the irradiated area (Fig. 7F). If the injection is made 2–3 h following PDT one notices that, although there is still no blood flow restriction to the treated area, the blood vessels appear constricted when observed from below the tumor/skin flap. At 24 h post-PDT, signs of hemorrhage within the tumor are more evident from both above and below the flap (Fig. 7, G, H). However, the major blood vessels surrounding and leading to the tumor appear intact (Fig. 7, G, I). Animals which exhibited the greatest degree of edema 24 h post-PDT usually showed signs of dye exclusion to the light treated area. As the edema subsides 48–72 h post-PDT, there is little if any sign of exclusion.

It is hypothesized that PII-PDT induced vascular occlusion is responsible for most tumor responses and that the effect is mediated by the release of thromboxane from platelets and/or endothelial cells (36, 37). In order to assess whether arachidonic acid metabolites are involved with EtNBS-PDT, we studied the therapeutic outcome from a combination treatment of phototherapy (conditions where 100% of animals were cured) and indomethacin (thromboxane inhibitor). All treated EMT-6 tumors (n = 9) responded to PDT (tumor dry weight of 8.6 ± 3.0 mg versus 35.0 ± 5.0 for nonirradiated) but only 33% were tumor free after 14 days.

DISCUSSION

The present in vivo evaluation of the photochemotherapeutic properties of EtNBS demonstrates its efficacy as a PDT drug. Furthermore, this study delineates several salient features of this photosensitizer which distinguish it from the majority of other photosensitizers currently being evaluated for the photodynamic treatment of malignant tissue.

On examining dye uptake/retention and distribution of EtNBS in the EMT-6 tumor model it is evident that the dye is rapidly taken up by various tissues including the tumor, maximizing at about 3 h post-s.c. administration and that the dye has a short half-life in these tissues being undetectable by absorption spectroscopy 24 h after its administration. Pharmacokinetic data (data not shown) also indicate that other benzophenoxazine derivatives behave in an analogous manner and that the dyes are also rapidly cleared from the liver, gallbladder, spleen, kidney, and brain within 24 h. This corroborates a study by Sloviter (38) which showed that a radioactive benzenophenoxazine derivative is cleared from these major tissues including the tumor in 24 h. The concentration of EtNBS which accumulates intratumorally during the 3-h period following s.c. dye injection is about an order of magnitude greater that what is seen with HPD (3) and other porphyrins (39, 40) in a variety of tumors during the same time period. Furthermore, a maximal discrimination between tumor and normal tissue (i.e., 4:1 for tumor versus skin) is achieved in only 3–8 h at which time PDT results in tumor cure without damage to the surrounding irradiated tissue. While several porphyrins can induce tumor cures following a short time interval between drug administration and light treatment (41, 42), it usually results in substantial damage to the normal surrounding tissues (43). In order to minimize photodamage to the normal tissue, the majority of porphyrin photosensitizers require a 24–48-h interval between drug administration and PDT (6, 44, 45).

Why the RIF tumor accumulates less dye than the EMT-6 is not certain. The fact that the in vitro data with these cell lines shows enhanced dye uptake compared to the EMT-6 cell line suggests that the tumor environment (vascular supply), composition (macrophage content, stroma composition, etc.), or rate of growth are affecting the accumulation in some way. It is known that the RIF tumor is less vascularized than the EMT-6 tumor (46). In agreement with our results, Nelson et al. (47) has also demonstrated a significant increase in the uptake and retention of HPD in the EMT-6 model as opposed to the RIF model via digital video fluorescence microscopy. However, Henderson et al. (29) did not find a difference in PII uptake between these same two tumor models.

It is not surprising that the i.p. injection of the dye results in approximately 50% less uptake compared with s.c. or i.v. administration to the EMT-6 tumor since these positively charged dyes have a strong tendency to adsorb to negatively charged sites on a variety of tissues (i.e., mesenteric connective tissues). This was verified by noting the extensive coloration of tissues in the peritoneal cavity of BALB/c as well as C3H/HeJ mice following dissection. The nonlinear relationship observed between administered dye concentration and tumor accumulation (Fig. 3) may, in part, be a consequence of this ionic binding or an indication that saturation of the tumor is occurring at higher dye concentrations. The fact that higher concentrations of administered EtNBS significantly increase the amount of dye in the blood plasma (Fig. 4) without causing a concomitant increase in tumor uptake implies that saturation is the more likely cause. Since only the 3-h time point was investigated in this study the matter can not be resolved without further pharmacokinetic data. The adsorbed dye at the injection site may constitute a slow release mechanism for the photosensitizer and thus substantially alter the pharmacokinetics.

Prior to discussing the PDT tumor response data it will be beneficial to briefly summarize the redox properties of EtNBS since they may govern its phototoxic efficacy. We have previously shown that EtNBS is capable of undergoing both an intracellular enzymatic reduction (dark reaction) and a light-induced reduction under anaerobic conditions which are reversible upon oxygenation (20). While this feature most likely influences the pharmacokinetics of the dye at both the cellular and systemic levels (20), the most important consequence of the reduction is the formation of a colorless, neutral compound (Fig. 8) which no longer absorbs light in the “therapeutic window” and therefore cannot function as a PDT agent. A ratio of oxidized to reduced dye will exist intracellularly at all times and will depend on the concentration of oxygen which is accessible to the microenvironment associated with the dye. The reversible photoreduction that is observed with these dyes should not be confused with the irreversible oxidative destruction (photobleaching) of porphyrin type photosensitizers seen during PDT (48, 49).

It has been well documented that HPD-PDT causes a depletion of ground state oxygen (O₂) in irradiated tissues (5, 50, 51). It has also been postulated that one factor responsible for the depletion of O₂ is the type II photooxidation of biomolecules since this consumes O₂ during the formation of ¹O₂. An approximate mathematical model for the PDT induced photochemical oxygen depletion has been proposed by Foster et al. (52–54). Their calculations suggest that reduced fluorescence rates increase the radius of oxygenated cells around capillaries and thus they are more effective in generating ¹O₂ throughout the
treated volume. This hypothesis is supported by their in vivo results. Since it is likely that a type II mechanism is also responsible for the phototoxic effects of EtNBS (16, 19), we expect a similar transient hypoxia to occur with EtNBS-PDT. Besides limiting the production of $^1$O$_2$, a significant decrease in the oxygen tension could shift the redox equilibrium to favor the colorless, leuko form of the dye. The occurrence of either of these events during EtNBS-PDT will influence the photodynamic efficacy.

As Fig. 5 indicates, decreasing the fluence rate increases PDT efficacy in the EMT-6 tumor model. More significant, the reduced fluence rate increased the number of nonpalpable tumors 14 days post-PDT. These results seem to further support Fosters’ basic premise. Although we have not investigated the effect of a fractionated light dose on tumor response, it would presumably lead to an enhanced photodynamic reaction through a similar mechanism. Others have shown that an enhanced PDT response does result from the use of a fractionated light delivery protocol with PII and phthalocyanines (5, 52).

Histological analysis of RIF and EMT-6 tumors 72 h post-PDT (7.5 mg EtNBS/kg; 50 mW/cm$^2$; 100 J/cm$^2$) revealed results which are not typically seen with porphyrin type sensitizers under conditions which lead to tumor cure (47). First there is minimal or no damage to the surrounding skin and peritumoral tissue. This is not a direct result of the discrimination in dye uptake between the tumor and skin as is evident from our skin response study in which BALB/c mice were given 60 mg of EtNBS/kg and photoradiated. At this high drug dose, the skin contained greater than 3 times the amount of dye in the treated tumor and yet no apparent edema, erythema, or damage occurred to the exposed skin. How is it possible that this high concentration of dye can leave the skin intact and yet one-third of this concentration in the tumor can lead to necrosis? This question may again be answered by considering the redox properties of the dye. The reduction of the EtNBS necessitates that it come in contact with intracellular reductases/dehydrogenases and their coenzymes NAD(P)H (20, 55–58) which are principally located in the mitochondria, endoplasmic reticulum, and the cytosol. Therefore, if a quantitative or qualitative difference exists between the enzymatic redox profiles of malignant and normal tissues it would not be surprising if a simultaneous change occurred in the ratio of oxidized to reduced dye between these two tissues. It has been well established that the metabolic changes which accompany the neoplastic transformation are mediated by altered enzyme profiles at these three intracellular sites (59–61). In addition, it has been proposed that the autofluorescence differences often seen between malignant and normal tissues (including skin) are the consequence of decreased levels of the reductant NADH in malignant tissue (41, 62). We should restate that in the presence of the electron donating coenzymes, NAD(P)H, the photoreduction of EtNBS can occur (20). In conjunction with these findings, Collier et al. (63) have shown that the skin of a variety of species rapidly reduce p.c. administered azo dyes and suggest that the reductive environment in skin serves to limit oxidative stress. We suggest that the skin is spared photodynamic damage as a result of a redox environment which leads to significant levels of the reduced, nonphotoactive form of EtNBS either prior to (dark reaction) or during PDT (photoreduction). This would constitute a novel method for increasing the photodynamic discrimination between tumor and normal tissues based on the inherent redox differences which exist in these tissue types. It should be emphasized that the extraction procedure which was used to quantitate the amount of dye in the tissues does not allow us to determine the ratio of oxidized/reduced EtNBS at the site of PDT because the extraction process itself results in the complete oxidation of dye. Therefore, one must be cautious when trying to equate the degree of PDT effects to the amount of dye extracted from the tissues. To reiterate, only the portion of dye which exists in the oxidized form in the tissue will be photoactive.

The diverse cellular metabolic microenvironments found in solid tumors as a result of the variability in tissue oxygen distribution, nutrient supply, pH, etc. (65) may also explain the viable cells dispersed throughout the tumor as well as around the periphery of the tumor mass following PDT. The fact that a rim of live cells sometimes occurs on the upper surface of the tumor, where the fluence is greatest, suggests it is not the result of inadequate light dosage. Because these diverse metabolic environments will display variable enzymatic activities, it is highly probable that they will also produce different ratios of oxidized/reduced EtNBS and thus be more or less susceptible to phototherapy.

Another fact that is readily apparent from the 72-h histology is the minimal damage to the vasculature (Fig. 7, H, I). While there is some stasis, hemorrhaging, and extravasation of RBC, it is substantially less than what appears necessary for a tumor response or cure using the majority of porphyrin photosensitizers (2, 66–68). Nelson et al. (47) recorded gross hemorrhage throughout EMT-6 tumors with HPD (10 mg/kg) and a light dose of 50 J/cm$^2$. The evidence to date suggests that complete and permanent vascular occlusion in the tumor as well as the surrounding normal tissue is a prerequisite for cures with the porphyrin type photosensitizers (2, 3, 5, 6). This correlates with the fact that a significant fraction of the tumor associated porphyrin is localized in the perivascular stroma and around blood vessels. The histological results seen with EtNBS 72 h post-PDT suggest that tumor necrosis may result primarily from intracellularly generated phototoxins and not from vascular occlusion. This is substantiated by several facts: (a) fluorescence spectroscopy reveals that the dye is in fact localized intracellularly; (b) in vivo PDT treatment leads to a 50–60% reduction in the viability of the cells isolated from the EMT-6 tumor immediately following PDT. In order for significant tumor cell death to occur in either the RIF or EMT-6 tumors under therapeutic conditions with PII, it was necessary for the tumors to remain in situ after completion of treatment (11, 29); (c) fluorescein dye exclusion experiments show that occlusion of blood vessels in the area exposed to light does not occur up to a minimum of 3 h following PDT. This circumstance ensures that the supply of oxygen is maintained to the tissue during the entire light delivery period. This is contrary to what occurs with the use of porphyrin type photosensitizers (2, 6, 40) which shut down the microvasculature during PDT and create a significant hypoxic cell fraction which can no longer respond to phototherapy. Sometimes, depending on the degree of edema, there is a partial constriction in the vasculature of the irradiated tissue during the 24–48-h period following phototherapy which most likely results from the hydrostatic pressure which is generated since a visual inspection of blood vessels leading to the tumor reveals that they are not injured. As
the edema subsides, fluorescein angiography indicates reperfusion of dye to the treated area. What part this temporary restriction in microcirculation plays in the treatment outcome of some tumors is uncertain at this time.

Since the determining factor for vascular photosensitivity appears to be the level of circulating photosensitizer (6), we examined the quantity of EtNBS in the blood plasma (Fig. 4) at the time of light delivery. At the concentration necessary for a high cure rate (7.5 mg EtNBS/kg = approximately 0.15 mg dye/mouse), only 54 ng of EtNBS (approximately 0.4% of the total injected dye) were present per ml of plasma. This is approximately 1000 times less than the quantity of PII present in the circulation of both humans and mice under similar circumstances (40, 69). In fact 1.0% of the injected dose of PII is still present in the circulation 24 h after its administration. In all likelihood, it is the rapid clearance of EtNBS from the circulation which accounts for the low levels of vascular damage in both tumor and surrounding skin. The initiating event(s) in porphyrin induced vascular occlusion is not well understood. However, there is experimental evidence (6, 37, 70) which strongly implicates the arachidonic acid metabolite thromboxane as a mediator of the vasoconstriction and platelet aggregation which is observed soon after PDT. Fingar and Wieman (37) have shown that the pharmacological (indomethacin) prevention of thromboxane synthesis totally inhibits the PII-PDT hemostasis and tumor (chondrosarcoma) response in Sprague-Dawley rats. Although all the BALB/c mice in our experiments responded to treatment with indomethacin/EtNBS, the decreased tumor cure rate (33 versus 100%) suggests that prostaglandins may play some role in the outcome of EtNBS-PDT-treated EMT-6 tumors.

Perhaps the most intriguing find from the 72-h histology of both the EMT-6 and RIF tumors is that 10–30% of the cells appear to be viable at this late time and yet both tumor systems have high cure rates. One could speculate that the immunogenic characteristics of the EMT-6 tumor (27) played a substantial role in its therapeutic outcome. However, the RIF tumor is reported to be nonimmunogenic (27) with as few as 10 viable cells leading to 50% tumor regrowth in the syngeneic host. Although speculative at this time, the possibility exists that either a specific acquired immune response towards the RIF tumor was stimulated by the photodynamic process or cells which participate in nonspecific effector mediated tumor eradication (i.e., macrophages, natural killer cells, lymphokine-activated killer cells, etc.) may be involved. The extensive photodestruction of the cancer cells with EtNBS may uncover normally nonexposed antigens. Such a phenomenon is observed with a complex branching glycolipid in which a branch is deleted, subsequently exposing a new antigenic determinant (71). The cellular damage is also expected to result in the subsequent release of a wide range of inflammatory and immune mediators. Both HDG and PII-PDT have been shown to release histamine, cincosanoids (prostaglandin E2), TNF, serotonin, interleukin 1β, and interleukin 2 (5, 6). Lymphokine activated killer cells in the presence of interleukin 2 can successfully mediate the regression of weakly immunogenic and nonimmunogenic tumors (72). While the majority of immunological studies with HDG and PII-PDT indicate a suppression of immune function (73), Yamamoto et al. (74) have shown that the use of extremely low concentrations of sensitizer, which precludes photodamage to the lymphocytes and the vasculature, leads to immunopotiation via the photovactivation of macrophages. This results in an enhanced Fc receptor mediated ingestion activity as well as the production of the cytokine, TNF. Since macrophages are antigen presenting cells, the ensuing phagocytosis of cancer cells may ultimately lead to the development of an immune response. Interestingly, an intact vasculature may be necessary for maximum immunopotientiation since Palladino et al. (75) have shown that vascularization is necessary for TNF to induce necrosis and regression in the Meth A sarcoma. Bellnier (76) reports that when TNF is administered i.v. prior to light exposure of mice bearing SMT-F adenocarcinomas, PII-PDT resulted in a statistically significant enhancement in tumor response. Although immunological involvement may be a plausible explanation for these PDT results, we cannot discount the possibility that the hematoxylin and eosin histological stain is not accurately delineating viable cells.

The present in vivo study demonstrates that EtNBS is a unique photodynamic agent which inactivates solid tumors (EMT-6 and RIF)primarily through direct tumor cell killing with a minimal disruption in the surrounding tissue or tumor vasculature. The dye is rapidly and preferentially accumulated in tumors in addition to being eliminated from most tissues within 24 h which ameliorates skin sensitization problems associated with long retention times. In addition, the unusual redox properties of EtNBS may amplify the phototoxic discrimination between neoplastic and normal tissue (i.e., skin). The ability to readily synthesize pure compounds which possess a wide range of photoactivities and physical properties should allow us to optimize the in vivo photochemotherapeutic efficacy of the benzophenothiazines.

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Novel Photodynamic Effects of a Benzophenothiazine on Two Different Murine Sarcomas

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