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Photodynamic Therapy of Disseminated Non-Small Cell Lung Carcinoma in a Murine Model

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Abstract

Background and Objective—Photodynamic therapy (PDT) of thoracic malignancies involving the pleural surfaces is an active area of clinical investigation. The present report aims to characterize a model for PDT of disseminated non-small cell lung carcinoma grown orthotopically in nude mice, and to evaluate PDT effect on tumor and normal tissues.

Study Design—H460 human non-small cell lung carcinoma (NSCLC) cells were injected percutaneously into the thoracic cavity of nude mice. HPPH-PDT (1 mg/kg, 24 h) was performed via the interstitial delivery (150 mW/cm) of 661 nm light to the thoracic cavity at fluences of 25–200 J/cm.

Results—H460 tumors exhibited exponential growth within the thoracic cavity consisting of diffuse, gross nodular disease within 9 days after intrathoracic injection. Tumor volume, measured by magnetic resonance imaging (MRI), was highly correlated with the aggregate tumor mass extracted from the corresponding animal. Intrathoracic PDT at fluences of 50 J/cm produced significant decreases in tumor burden as compared to untreated controls, however mortality increased with rising fluence. Accordingly, 50 J/cm was selected for MRI studies to measure intra-animal PDT effects. Tumor distribution favored the ventral (vs. dorsal), caudal (vs. cranial), and right (vs. left) sides of the thoracic cavity by MRI; PDT did not change this spatial pattern despite an overall effect on tumor burden. Histopathology revealed edema and fibrin deposition within the pulmonary interstitium and alveoli of the PDT-treated thoracic cavity, as well as occasional evidence of vascular disruption. Prominent neutrophil infiltration with a concomitant decline in the lymphocyte compartment was also noted in the lung parenchyma within 24 hours after PDT.

Conclusion—HPPH-PDT of an orthotopic model of disseminated NSCLC is both feasible and effective using intracavitary light delivery. We establish this animal model, together with the

treatment and monitoring approaches, as novel and valuable methods for the pre-clinical investigation of intrathoracic PDT of disseminated pleural malignancies.

Keywords

HPPH; Photochlor®; 2-[1-hexyloxyethyl]-2-devinyl pyropheophorbide-a; interstitial illumination; magnetic resonance imaging; non-small cell lung carcinoma; photodynamic therapy; pleural malignancy

INTRODUCTION

Applications of photodynamic therapy (PDT) in the treatment of diffuse superficial disease, for example in the thoracic or peritoneal cavities, has been met with varying degrees of success (1-6). Challenges in the delivery of PDT to such irregularly shaped and complex surfaces include heterogeneities in disease burden, photosensitizer uptake, and light distribution, among other factors (7-9). Furthermore, pre-clinical evaluation of approaches to mitigate or modulate these parameters has been limited by the lack of an animal model and approach suitable for studying PDT for such diffuse diseases.

Orthotopic animal models of human malignancies have become a standard in translational investigations due to their biological characteristics, which are more relevant to human disease than tumors grown by heterotopic transplantation (10,11). It is therefore not surprising that tumors grown at an orthotopic location respond differently to therapeutic intervention than their subcutaneous counterparts. For example, a recent study of murine models of lung cancer (12) reported that subcutaneously-propagated tumors exhibited more hypoxia than orthotopic or spontaneous models of the same cell lines; correspondingly, these subcutaneous tumors were more responsive to a hypoxia-targeted cytotoxin. Chen et al (13) compared the effect of subcutaneous versus orthotopic location on the microenvironment and PDT response in rodent prostate tumors. The orthotopic tumors demonstrated more rapid growth, more dense inter-vascular spacing, and less hypoxia than their subcutaneous equivalents. This resulted in better PDT response at the orthotopic location as a function of the treatment protocol used. Orthotopic and subcutaneous tumors responded similarly to a vascular-targeting PDT protocol (15 minute drug-light interval with verteporfin), but a protocol that utilized a longer drug-light interval to create direct tumor cytotoxicity was significantly more effective in the better-oxygenated orthotopic setting. Thus, as for other therapeutic modalities, there is clinical relevancy in the pre-clinical evaluation of PDT at orthotopic locations. In addition to prostate, tumors grown orthotopically at sites including brain, lung, bladder and peritoneal cavity (14-19), have been studied for their responses to PDT.

Although it has been established that definitive treatment for early stage and locally advanced non-small cell lung carcinoma (NSCLC) should consist of any combination of surgery, chemotherapy, and radiation therapy, treatment of pleurally disseminated NSCLC has generally been palliative as illustrated by the abysmal 6-9 month median survival rate (20-22) and recent upstaging from T4 to M1a disease (23). In an attempt to improve local control and consequently overall survival, a recent trend in more aggressive therapies has been considered including chemotherapy (24,25), surgical resection (26-30), and/or PDT (1,3,5,6,31). This is important, as pleural spread of NSCLC occurs in 10-50% of patients during the course of lung cancer (32). In an effort to study M1a-staged Non-Small Cell Lung disease (AJCC, 7th ed.) involving pleurally disseminated disease without any interference from intraparenchymal or metastatic sources, we intrathoracically introduced human cells that were originally derived from the pleural fluid of a person with non-small cell lung cancer (33).

In the clinic, several different photosensitizers have been evaluated in trials of PDT of disseminated lung malignancy, including both Photofrin and mTHPC (m-tetrahydroxyphenylchlorin) (1,5,6). More recently, though, HPPH (Photochlor®; 2-[1-hexyloxyethyl]-2-devinyl pyropheophorbide-a) has been used in an ongoing Phase I trial at our institution with the objective of establishing the maximum tolerated doses of photosensitizer and activating light for PDT of maximally-debulked pleural malignancies. As such, HPPH was selected for the present study with its dose and drug-light interval (1 mg/kg, 24 h) chosen based on pre-clinical demonstration of efficacy (34), as well as accumulation of drug in lung tissue (35). The selected illumination conditions (25 –200 J/cm at 150 mW/cm) approximate those previously studied in clinical applications of interstitial PDT (36). This trial employed a different photosensitizer than our current investigations but nevertheless establishes 150 mW/cm as a reasonable clinical fluence rate for interstitial light delivery. A fluence rate of 150 mW/cm was desired because it could be expected to produce irradiances within the tissues of the murine thoracic cavity that are at least as high as those experienced during the Phase I clinical trial of pleural malignancies, albeit in both situations these irradiances can be expected to vary significantly within the patient or animal as a function of the light source position and light scattering and absorption within the thoracic cavity.

The goal of the present report was to model PDT treatment of disseminated thoracic disease. The percutaneous intrathoracic introduction of H460 human non-small cell carcinoma (NSCLC) cells in nude mice (37) led to the development of diffuse disease incorporating the pleural surfaces. As successfully performed for the treatment of disease lining airspaces (e.g. in endobronchial applications (38)), thoracic PDT was delivered through the interstitial placement of a cylindrical diffusing fiber (1 cm in active length). The resulting effects on tumor burden and normal tissues of the thoracic cavity are described. Finally, using MR (magnetic resonance) imaging, we evaluated the distribution of tumor burden within the murine thoracic cavity, as well as PDT effect on this burden within an individual animal.

METHODS

Tumor model

H460 large cell lung cancer cells (ATCC, Manassas, VA) were cultured in RPMI-1640 medium (ATCC) supplemented with 10% fetal bovine serum (Gibco, Carlsbad, CA), 2 mM L-glutamine (Gibco), 100 units/ml penicillin (Gibco), 100 µg/ml streptomycin (Gibco) and maintained in a humidified atmosphere with 5% CO₂ at 37 °C. Cells in log phase were harvested, and resuspended at 1 ×10⁶ cells/ml in a 1:1 solution of phenol red-free matrigel basement membrane matrix (BD Biosciences, San Jose, CA) and normal saline. Ketamine/xylazine-anesthetized 7-9-week-old athymic Ncr-nu/nu female mice (NCI-Frederick, Frederick, MD; Taconic, Hudson, NY) were injected with the cell suspension percutaneously through the intercostal muscle into the right lateral thorax at the dorsal axillary line approximately one centimeter above the caudal edge of the ribcage as previously described (37). Animals were under the care of the University of Pennsylvania Laboratory Animal Resources. All studies were approved by the University of Pennsylvania Institutional Animal Care and Use Committee.

Photodynamic therapy

Photodynamic therapy was performed using the photosensitizer, HPPH (Photochlor®; 2-[1-hexyloxyethyl]-2-devinyl pyropheophorbide-a), kindly provided by B.W. Henderson and T.J. Dougherty (Roswell Park Cancer Institute, Buffalo, NY). The dry powder of HPPH was formulated as a stock solution (~500 µg/ml) in 5% dextrose (in water) with 2% ethyl alcohol (95%), and 1% polysorbate 80; adjusted to pH 7.3-7.5 using 0.1 M sodium carbonate.

Frozen aliquots were defrosted and further diluted in D5W to a final concentration of 0.1 mg/ml. Mice were injected with 1 mg/kg HPPH (via tail vein) and ~24 h later interstitial illumination of the thoracic cavity was accomplished using a customized cylindrical diffusing fiber (active length of 1 cm; diameter 200 μ m; Rare Earth Medical, Inc., West Yarmouth, MA). In anesthetized mice (isoflurane with medical air; VetEquip anesthesia machine, Pleasanton, CA) the cylindrical diffusing fiber was guided into position through the bore of a 19-gauge needle that had been inserted ~1.2 cm into the right side of the thoracic cavity as described for tumor cell inoculation. The needle was withdrawn by sliding it along the external portion of the fiber, leaving the 1-cm active length of the fiber within the cavity. Illumination was performed using a 661-nm diode laser (B&W Tek, Inc., Newark, DE) at a fluence rate of 150 mW/cm and the indicated fluences. An integrating sphere (Diomed, Inc., Andover, MA) was used to measure power output from the fiber.

Magnetic resonance imaging

Anesthetized animals were imaged by magnetic resonance imaging using a 9.4 Tesla horizontal bore MRI system equipped with a 12 cm ID gradient tube capable of achieving a gradient amplitude of 40 G/cm in 200 μ sec (Magnex Scientific, Abingdon, UK) interfaced to a DirectDrive console (Agilent Technologies, Palo Alto, CA). Animals were mounted in a 35 mm ID quadrature birdcage coil (M2M Imaging Corp., Cleveland, OH) and maintained under anesthesia by inhaled isoflurane with medical air. Vital signs were monitored throughout imaging with a rectal temperature probe, pneumatic pillow, and two-lead subdermal EKG, all interfaced to a MRI-compatible vital signs monitoring system (SA Instruments, Inc., Stony Brook, NY). Core body temperature was maintained at $36\pm 1^\circ\text{C}$ with a regulated warm air source directed over the animals while in the magnet. The heart rate was maintained above 350 beats per minute and the respiratory rate at 50-80 respirations per minute by manually adjusting the isoflurane concentration delivered through the nose cone. Following acquisition of scout images, a contiguous series of respiration- and cardiac-gated spin echo images spanning the entire lung cavity were acquired during post-expiratory periods (TR (repetition time) = 500-2000 ms, TE (echo time) = 11 ms, FOV (field of view) = 30 mm $\times$ 30 mm, slice thickness = 0.5 mm, matrix = 256 $\times$ 256, slices = 40-50, 2 averages). Separate acquisition scans were serially acquired in the axial, sagittal, and coronal planes.

Visualization and quantitative analysis of MR images were performed using MIPAV software (available at <http://mipav.cit.nih.gov/index.php>) after first registering in ImageJ software (available at <http://rsb.info.nih.gov/ij>) the multislice image file from the individual slices spanning the entire thoracic cavity. Regions of interest (ROI) were manually drawn around the areas of visible thoracic opacities, representing tumors, in each image using MIPAV. "Tumor" was distinguished from normal structures based upon the hyperintensity appearing on MRI. An aggregated tumor area was calculated, and multiplied by the slice thickness (0.5 mm) to obtain the total tumor volume in each mouse.

Assessment of tumor growth

At indicated time points relative to inoculation of tumor cells, animals were euthanized in a CO₂ chamber and examined for tumor burden by median sternotomy. Digital photographs were used to document spatial tumor distribution followed by complete extraction ("cherry-picking") of all gross tumor nodules. To establish a growth curve, the total tumor mass was recorded for each animal and plotted versus time from inoculation. In a subset of these animals, MRI was performed prior to euthanasia for comparison of weighed tumor burden with total tumor volume (as calculated from MRI).

Assessment of tumor response to PDT

At 12 days after tumor cell inoculation, animals were treated with PDT (described above), defined as “day 0”. Response as a function of light fluence over the range of 25 – 200 J/cm was assessed by animal euthanasia four days later (defined as “day +4”) followed by dissection and weighing of tumor burden. Tumor weight was compared among control (no light or drug, drug-only, light-only) animals and those treated at each light fluence. To assess tumor response within individual animals, MRI was performed on day –1 and day +5 relative to light delivery (50 J/cm). The relative change in tumor volume (volume on day +5/ volume on day –1) was calculated for each animal and compared among the control and treated groups.

Assessment of tumor distribution

In both control and PDT-treated mice, the distribution of tumor burden among spatially-defined bisections (ventral vs. dorsal, caudal vs. cranial, right vs. left) of the thoracic cavity was calculated from the MR images using custom programs written in MATLAB (MathWorks, Natick, MA). Anatomic landmarks used to establish borders consisted of the sternum-spinal cord line (sagittal plane), right to left mid axillary lines (coronal plane), and midpoint to the apical thorax and diaphragm (axial plane). Based on these boundaries, the thoracic cavity was divided into the above-indicated bisections for the three separate planes, and the amount of tumor burden in each half was computed using the area of tumor ROI encompassed within its respective anatomic compartment. Volumes were calculated from the areas by multiplying the calculated area by slice thickness (0.5 mm), as described in *Magnetic resonance imaging*.

Pathological evaluation

Collection of organs, including heart, lungs and liver, as well as whole blood was performed in mice with and without intrathoracic tumors at indicated time points relative to PDT. Mice were surgically anesthetized with ketamine/xylazine and blood was collected by cardiac puncture for later analysis of hematology and serum chemistries (Antech Diagnostics GLP, Morrisville, NC). After confirmation of death via exsanguination, the thoracic cavity was opened, and lungs were insufflated through the trachea with 10% neutral buffered formalin. The right and left lungs were excised with the heart and liver. Samples were fixed in formalin for at least 48 hours, and then embedded in paraffin. Sections were cut from these blocks at 5- μ m intervals for 3 levels, stained with hematoxylin and eosin, and examined by a veterinary pathologist (A.D.) blinded to the treatment groups.

Statistical analysis

Continuous outcomes were characterized by descriptive statistics, i.e., the mean, median, standard deviation, and range. Ordinal comparisons of PDT fluences according to mouse mortality or tumor mass, as well as comparisons of control versus PDT effect on relative tumor volume (MRI data), were performed by the Kruskal-Wallis or two-sample Wilcoxon rank-sum (Mann-Whitney) tests. Assessment of the relationship between mortality and increasing fluence was estimated using logistic regression. Hematological data were compared using the Wilcoxon rank-sum test. To assess tumor growth rate using an exponential model, we fit a linear model to the log-transformed volume data using time as the predictor. The results of this model are presented using the exponentiated form of the fit of this model.

To assess spatial differences in tumor distribution at baseline (day –1) and after PDT (day +5), we fit a linear mixed-effects model to the volume data in order to determine the effect of location (ventral vs. dorsal, caudal vs. cranial, and right vs. left) while accounting for

correlation between repeated measures on the same animal. The data were log-transformed for this analysis to approximate normality. Using the day +5 data, the effect of PDT was estimated in comparison to a combined control group (light-only or drug-only). This essentially used an analysis of covariance approach such that the day +5 model was adjusted for volume at baseline at each site.

In order to determine whether PDT effects were location-specific, we compared the fit of two different models to the day +5 data. Both models incorporated the effect of PDT on tumor burden, but one model incorporated it in a location-independent manner while the other incorporated specific location effects, using three interaction terms between PDT effect and the individual positions. The two models were compared using a likelihood ratio test, with the results indicating that a location-specific model did not significantly improve the fit compared to the location-independent model. Relative differences were assessed using a Wald test. The results of the location-independent model are accordingly reported in the Results.

For all tests, a type I error rate was set at 0.05. The spatial distribution of tumor volume was analyzed using the *nlme* package in R 2.12 (R Foundation for Statistical Computing, Vienna, Austria (39)), while other analyses were performed in STATA 11.2 (StataCorp, College Station, TX) or JMP 9 (SAS, Cary, NC). Graphs were generated in R (library ggplot2) (40) or STATA.

RESULTS

Characteristic appearance and growth of thoracic disease

Within 9 days of the intrathoracic injection of H460 cells, multinodular disseminated intrathoracic masses with a serosanguinous pleural effusion were evident at necropsy by gross examination (Fig. 1a). The disease was confined to the thoracic cavity bilaterally and the nodules appeared secured to the parietal pleura of the diaphragm, heart and mediastinum, lungs, and rib cage as expected (37). Nodules displayed a grape-like growth pattern attached to one another by fibrous bands, rather than free-floating in the thoracic cavity. Although the lungs rarely contained gross disease visible at necropsy, histopathologic analysis revealed neoplastic nests within the pulmonary parenchyma (Fig. 1b). Multiple neoplastic foci were also observed in the adjacent pleural tissue, including the mediastinal brown fat.

Tumor growth rate was determined through weighing tumor burden in separate groups of animals sacrificed between 9 and 20 days after tumor cell inoculation. An exponential relationship existed between total days of tumor propagation and aggregate tumor mass (Fig 1c), which is in accordance with the well-described exponential *in vivo* solid tumor growth kinetics (41). Based on this exponential model, a 12-day period was chosen to allow for sufficient tumor growth prior to performing PDT, and assessment of PDT effects at 4 days (by tumor weight) or 5 days (by MRI) after light delivery. This time frame represents a relatively linear portion along the tumor growth curve that was not associated with morbidity. Notably, the thoracic cavity is capable of harboring at least an 11-fold proliferation in tumor mass between day 9 and 20 after tumor inoculation, but not without the associated development of significant respiratory distress.

Fluence dependence of intrathoracic PDT response

The effect of intrathoracic PDT on tumor burden was examined using the photosensitizer HPPH (1 mg/kg, 24 h drug-light interval) and interstitial illumination (661 nm) over fluences of 25 – 200 J/cm delivered at 150 mW/cm. Due to the technical complexities of interstitial fiber placement and subsequent illumination of the thoracic cavity, initial PDT studies focused on establishing the feasibility and associated morbidities of light delivery to

the thoracic cavity of tumor-bearing animals. Interstitial introduction of a cylindrical linear light source through the bore of a 19-gauge needle provided a reliable means of inserting the otherwise flexible light fiber within the thoracic cavity. The process of intrathoracic fiber placement led to acute morbidity (within 5 minutes) in ~11% of animals, which was attributed to a technical etiology likely the result of puncturing the heart or great vessel with the needle tip, as the mouse died relatively quickly with profuse exsanguination. PDT itself was associated with a ~4.5% acute mortality rate that was again deemed to be a result of fiber positioning since death occurred during or immediately after light delivery, independent of the fluence delivered. Over periods of more extended observation (through 4 days after PDT), fluence-dependent morbidity was evident and resulted in mortality rates of 8%, 11%, 38%, 54%, and 50% at fluences of 25, 50, 80, 150 and 200 J/cm, respectively. In contrast, light delivery in the absence of photosensitizer did not result in any animal deaths. Using logistic regression, we estimated that each 25 J/cm rise in fluence was associated with a 1.9-fold (95% CI: 1.5, 2.4, $p < 0.0001$) increase in the odds of death.

Having established the feasibility of intrathoracic light delivery, PDT effects on tumor burden were studied at 4 days after illumination for light fluences of 25–200 J/cm. A significant reduction in tumor mass (mean $\pm$ SD), compared to control mice (0.264 $\pm$ 0.081 g) unexposed to photosensitizer or light, was apparent at light fluences of 50 J/cm (0.111 $\pm$ 0.057 g, $p < 0.0001$), 80 J/cm (0.116 $\pm$ 0.074 g, $p < 0.0001$), 150 J/cm (0.083 $\pm$ 0.045 g, $p = 0.001$), and 200 J/cm (0.113 $\pm$ 0.006 g, $p = 0.024$) (Table 1). The high fluence (150 and 200 J/cm) groups do not contain as many animals as the other fluence groups because of the high mortality rate associated with these conditions and a consequential decision to discontinue investigation of these high fluences after an initial set of studies. Furthermore, the lowest fluence studied, 25 J/cm, was insufficient at promoting a decline in tumor mass (0.246 $\pm$ 0.102, $p = 0.689$). Also, mice treated with HPPH-only or light-only demonstrated the same amount of tumor burden as the drug- and light-free control on day 4 after PDT. Because there was no statistically significant difference in tumor mass between animals treated at 50 J/cm vs. 80 J/cm ($p = 0.793$), but fewer animal deaths occurred at the 50 J/cm fluence ($p = 0.048$), the lower fluence was chosen for subsequent studies.

In situ PDT reduces intrathoracic tumor burden

In order to assess the effect of PDT on intrathoracic tumor burden at the level of the individual mouse, animal imaging by MR was utilized to compare tumor burden before and after PDT.

These studies first involved establishing that tumor volume imaged by MR was representative of tumor mass, measured by collection after animal euthanasia. Respiratory- and cardiac-gated spin echo MRI spanning the entire thoracic cavity during post-expiration was performed; on the resulting image slices, tumor boundaries were manually drawn based on hyperintensity values (Fig. 2). Within several hours of completing MRI, mice were euthanized and tumor burden was collected. A linear relationship existed between weighed tumor mass and imaged tumor volume on axial (Fig. 3a; $r^2 = 0.81$), coronal (Fig. 3b; $r^2 = 0.85$), and sagittal (Fig. 3c; $r^2 = 0.88$) planes. It was also reassuring that no MRI planes contained evidence of extrathoracic disease, as was previously established by necropsy.

Subsequent to this validation, treatment effect on tumor volume was measured in control and PDT-exposed mice imaged by MR 24 hours prior to light administration (day -1) and again at five days (day +5) after receiving PDT. A fluence of 50 J/cm was chosen based on the above experiments, and as found in these studies, light-only did not produce a discernable difference in tumor response (left panel, Fig. 4). In contrast, PDT resulted in a significant reduction in tumor burden compared to the controls (right panel, Fig. 4). At 5 days after light delivery, the tumor volume in PDT-treated mice increased (mean $\pm$ SD) by a

factor of 2.2 ± 0.3 , which was significantly less ($p = 0.04$) than the $3.4\text{-fold} \pm 0.7$ increase in control mice, thereby providing evidence that PDT slowed disease progression.

Spatial distribution of tumor growth and PDT response

Due to the diffuse nature of this disease, we were interested in examining whether a spatial pattern of tumor burden existed within the thoracic cavity. For this purpose, tumor volume was quantified from the MR images as a function of location within the thoracic cavity (i.e. ventral vs. dorsal, caudal vs. cranial, and right vs. left). Significant differences in initial tumor burden were detected as a function of location (Table 2). On the day prior to PDT (day -1), tumor volumes were 1.73-fold higher in the ventral vs. dorsal side of the cavity, 1.63-fold higher in the caudal vs. cranial side of the cavity, and 1.3-fold higher in the right vs. left side of the cavity. Thus, the largest burden of disease was localized to the right caudal ventral compartment of the thoracic cavity by a factor of 3.7 ($1.30 \times 1.63 \times 1.73$) times larger than the smallest tumor burden compartment (left cranial dorsal segment of the thoracic cavity).

In PDT-treated animals, mean tumor burden on day +5 decreased to 80% (95% CI: 66%, 95%) to that of control-treated animals ($p = 0.019$) without detection of a differential PDT effect among the investigated locations ($p = 0.36$). Accordingly, the PDT effect was incorporated into the model in a location-independent fashion and the distribution of tumor volume was considered for the day +5 time point. Not surprisingly, the right caudal ventral thoracic cavity compartment continued to be the preferential location of tumor burden by an overall factor of 1.9 [factors of 1.15 (right), 1.17 (caudal), and 1.38 (ventral) compared to their opposite sides, respectively]. Comparison of right versus left side approached, but did not achieve strict statistical significance at the five-day time point ($p = 0.054$).

Local normal tissue damage and systemic effects

Intrathoracic PDT to 50 J/cm was delivered to tumor-free mice so as to determine PDT effect on normal tissues. Animals treated with HPPH-only (drug controls), as well as untreated (no HPPH or light), generally exhibited normal histology (Fig. 5a) except for occasional mild interstitial congestion that likely resulted from anesthesia/euthanasia. In the animals that received PDT, damage to the lung was present in the interstitium as vascular congestion, fibrin deposition, and neutrophil infiltration. The alveoli contained edema, fibrin, neutrophils, as well as scattered necrotic cellular debris. Occasional blood vessels were characterized by thickened walls comprising fibrin, neutrophils and perivascular edema (Fig 5b). In mice that received light-only (no photosensitizer), similar changes were noted within the blood vessel walls; however, the interstitial and alveolar changes were milder, and no necrotic debris was identified. Furthermore, no differences were noted between the right and left lung in any of the mice.

After PDT, the heart demonstrated moderate acute inflammation of the epicardium (the outmost layer of the heart), characterized by fibrin, edema and neutrophilic infiltration, and similar mild changes of the subjacent myocardium. Notably, treatment with light-only yielded similar pathologic changes of the epicardium as were found in the PDT-treated samples. Furthermore, more damage was located in the right atrium compared to the left side of the heart in both the PDT- and the light-treated groups. The predominance of superficial right atrial damage in the context of similar response to light-only and PDT, points to a role of fiber proximity to the heart as the major source of light-induced damage (irrespective of the presence of photosensitizer). Hearts from a control (no HPPH nor light) animal, as well as those that received only HPPH, were free of significant lesions.

Serum chemistries (Table 3) revealed that an acute systemic inflammatory response was triggered within 24 hours of PDT as characterized by significant increases in percentages of circulating neutrophils and monocytes with a concomitant decrease in the percentage of lymphocytes. Taken together with the trend of decreased hemoglobin concentrations within the first 24 hours of light administration, it appears that PDT-treated animals developed “anemia of chronic disease” due to a treatment-induced pneumonia. This is further supported by the considerable thrombocytopenia (decrease in circulating platelets) observed within 24 hours after PDT, which likely resulted from inflammatory-mediated platelet consumption.

PDT led to insignificant increases in BUN (blood urea nitrogen) but had no effect on creatinine levels, suggesting normal kidney function. The liver enzymes, AST (aspartate aminotransferase) and ALT (alanine aminotransferase), were exceptionally elevated in PDT-treated mice (compared to controls), despite no histopathologic abnormalities of the liver detected in any control or treated animals. Potential etiologies include light scatter to the surface of the liver (which could cause an acute rise in hepatic enzymes without pathologic changes) and/or cardiac or lung damage (as AST and ALT are not specific to liver enzymes). The latter may be a more reasonable explanation in the face of pathologic changes to the epicardium in combination with elevated CPK (creatine phosphokinase) levels that are consistent with muscle damage from the delivery of light to the thoracic cavity.

DISCUSSION

Although PDT for superficial, diffuse disease is a challenging application, it possesses substantial clinical potential for disseminated malignancies including those of the pleural and abdominal surfaces (42,43). Similarly, PDT of diffuse disease in a small animal, especially within the thoracic cavity, is complex, thereby limiting the development of pre-clinical models for this application. We demonstrate that PDT can be delivered to the mouse thoracic cavity via a cylindrical diffusing fiber with therapeutic fluences and acceptable morbidity. Furthermore, we exploited the benefits of small animal imaging with a non-ionizing radiation modality, that being MRI, to quantify the spatial distribution of tumor burden and investigate PDT effects by location within the murine thoracic cavity. These data serve to establish this animal model, together with the treatment and monitoring approaches, as methods with significant potential value for the pre-clinical investigation of intrathoracic PDT for disseminated pleural malignancies.

The orthotopic model of the present report was adapted from that described by Onn et al (37), who surveyed a panel of NSCLC cell lines and a small cell lung carcinoma cell line for their tumorigenicity and growth characteristics after intrathoracic injection. This method was chosen because it can be established without surgery, which limits morbidity prior to PDT, and it is associated with diffuse pleural spread, which is relevant to clinical PDT of pleural disease. In our observations of intrathoracic H460 proliferation, dissemination and pleural effusion were generally present within 9 days of the injection of 1×10^6 cells. Microscopic lesions within the lung itself were readily visible at histopathological evaluation. However, when compared to the cell lines studied by Onn et al (37), we found that intrathoracic dissemination of H460 tumor cells progressed more rapidly and/or was aided by independent pleural seeding.

Few pre-clinical models of intrathoracic PDT of orthotopic lung disease currently exist. Among those established, most involve *localized* light delivery to the diseased lung in conjunction with thoracotomy. For example, Opitz et al (44) reported that focal illumination of localized subpleural mesothelioma nodules in rats resulted in 50-55% necrosis of tumor

when treated with mTHPC and 80-88% when treated with verteporfin. The underlying aorta, bronchus and esophagus were spared from damage; however drug doses that produced the greatest amount of tumor necrosis also led to fibrosis in adjacent normal lung tissue. From these results, the authors concluded that focal intracavitary PDT in the rat was well tolerated, and a similar illumination approach was subsequently applied for PDT-enhanced uptake of the chemotherapy drug, Liporubicin, in sarcomas of the rat thoracic cavity (45).

In contrast to the above results with focal lung illumination, the evaluation of pneumonectomy followed by spherical illumination to treat diffuse thoracic disease proved fatal to all animals studied (18). Thus, the results of the present report are significant in that they establish that intracavitary light delivery to the murine thoracic cavity is feasible, and well-tolerated with minimal mortality. The improved mortality rate we experienced at therapeutic fluences is undoubtedly related to the lack of accompanying surgery, but nonetheless, control of tumor progression was still demonstrated. It is also important to note that different photosensitizers were used in these studies; our work utilized HPPH while Krueger et al (18) used mTHPC, which could account for differences in outcome.

HPPH-PDT of the murine thoracic cavity can result in significant numbers of animal deaths as evidenced by a mortality rate of 50% at the highest fluences tested; however, a dose of 50 J/cm resulted in a more modest mortality rate of 11%. As noted in the Results, fluence-dependent mortality was considered separately from mortality related to placement of the light fiber itself. This distinction is important because fluence-dependent mortality is inherent to the study, and as suggested by histopathology, likely attributable to PDT effects on normal lung tissue (including pneumonia and vascular congestion, the severity of which can be expected to increase with increasing fluence). In contrast, technical mortality due to light fiber insertion or positioning is potentially addressable, for example through ultrasound guidance of fiber insertion or increasing operator experience with the technique.

Damage to the normal tissues, although undesirable, is inevitable in PDT of large surface areas with untargeted photosensitizers. The histopathologic changes associated with normal tissue injury in this study are consistent with that reported by others in thoracic PDT of tumor-free animals. Interstitial light delivery via a bare-tipped fiber in mTHPC-PDT of rat lung led to vascular damage, necrosis, RBC extravasation, edema, and fibrin deposition in the treated foci, as well as neutrophil infiltration into the surrounding areas (46). Similar evidence of PDT-induced vascular disruption, alveolar congestion and hemorrhagic necrosis was noted in the lungs of mTHPC-treated pigs (47) and Photofrin-treated rats (48). Unlike that noted in the studies by Fielding et al (46,47), however, our evaluation of interstitial HPPH-PDT of the mouse thoracic cavity did not create marked zones of necrosis, but rather scattered foci of necrotic cells. Treatment-induced pneumothorax, as has been detected in other studies (47) could provide one explanation of this finding as it would alter the geometry of light exposure to the lung, while theoretically increasing diffuse dose to the pleural surfaces. Interestingly, diffuse light delivery via spherical illumination after pneumonectomy in the rat also did not produce identifiable focal zones of necrosis (18), which reconciles well with the results of our study.

Local PDT damage to organs of the thoracic cavity manifested as an acute systemic inflammatory response, characterized by massive increases in circulating neutrophils and macrophages, decreases in blood lymphocytes, and thrombocytopenia. Such an inflammatory response is a well-documented characteristic of PDT, including applications that utilize HPPH or Photofrin as photosensitizers (49,50). Furthermore, elevations in AST, ALT and CPK were also noted within 24 hours after thoracic PDT, all of which could be a consequence of acute damage to lung or muscle tissue (51). Elevations in CPK have also been found after Photofrin- or HPPH-PDT of canine lung (52,53) and returned to normal

within a week of treatment (53). Importantly, in the present study, no biochemical evidence of a systemic response was found after treatment with light-only despite its association with limited damage to the lung interstitium and vasculature, most likely attributable to physical or thermal damage of tissue in close contact with the light fiber. Similarly, light-only controls produced no effect on tumor weight or volume compared to untreated and/or HPPH-only controls. This further indicates that local histopathologic damage from light (alone) was too inconsequential to produce a more generalized effect.

MRI has been used to detect primary tumor as well as metastases to the rodent lung (54-56) and provides the advantage of assessing treatment effect in the same animal. Although imaging the murine lung can prove challenging due to organ motion (57), it is feasible, especially with the benefit of respiratory and cardiac gating (58-60). Our initial studies establish that tumor volume measured by MRI is highly correlated with the mass of tumor collected and weighed at necropsy, and thereby provides a good representation of total tumor burden. Imaging was then applied to measure the intra-animal change in tumor burden between the day before (day -1) and five days after (day +5) PDT. Focus was placed on these time points as opposed to imaging more frequently because we had found that the repeated physiological stress of prolonged anesthesia (at least 1 hour from setup to acquisition of all three imaging planes within one mouse) on animals with lung disease led to their demise immediately after MRI. As an aside, a more slowly growing tumor model would enable the imaging to be scheduled less frequently and could perhaps facilitate multi-time point monitoring over a longer duration after PDT.

MRI results showed PDT to significantly slow tumor progression, with the PDT-treated tumors increasing in volume by 2.2-fold between day -1 and day +5, compared to a 3.4-fold increase in the control tumor volume. This increase in tumor volume in control mice is in good agreement with the tumor growth curve, which predicted an approximately four-fold increase in tumor burden between 11 days (corresponding to day -1) and 17 days (corresponding to day +5) after injection of the tumor cells. The presence of a significantly smaller increase in disease burden in the PDT-treated mice suggests that PDT reduced growth rate, which is supported by the histologic observation (data not shown) that fewer mitoses per high power field were present in PDT-treated tumors (5-9 mitoses) than in the light-treated controls (13 - 17 mitoses). Because this tumor model is highly aggressive and grows rapidly, evidence of a PDT-created reduction in tumor burden would best be detected within the first one or two days after treatment. That said, the presence of inflammation complicates interpretation of MR images within the first several days of treatment, so day +5 was instead used as the post-treatment imaging time point. At 24 h after PDT, necrosis is detectable via histologic assessment, but the presence of pre-existing necrosis invalidates any attempt to estimate treatment-induced tumor necrosis. Moreover, the presence of pre-existing necrosis suggests that these tumor nodules contain areas of hypoxia, which could reduce therapy-induced cell death by limiting delivery of photosensitizer and creation of the oxygen-dependent reactive species responsible for PDT-induced damage. Such hypoxic areas are, indeed, characteristic of clinical lung malignancies (61,62). Continuing studies will more closely examine tumor hypoxia and any limitations it imposes on PDT-created cell death, as well as the introduction of approaches that augment direct cellular damage when combined with PDT.

A second advantage to MR imaging is the ability to provide information on the spatial distribution of tumor within the thoracic cavity. Our analyses discovered initial tumor growth to favor the right caudal ventral compartment of the thoracic cavity, which is most logically explained by injection of the tumor cells into the right side of the cavity and the effects of gravity as the tumor disseminates. PDT significantly reduced tumor progression, but it had no substantial impact on tumor burden distribution. This suggests that the

treatment light was scattered throughout the cavity, and therefore no spatial compartment was completely spared by PDT. More generally, the results serve to prove this analytic approach as useful in quantification of heterogeneities in tumor distribution within the murine thoracic cavity. In clinical applications of PDT for disseminated pleural disease, it is necessary to overcome heterogeneities in the distribution of millimeter-sized nodules in order to control disease burden; this makes it both relevant and valuable to assess new PDT approaches in pre-clinical models for their ability to reduce not only overall disease burden but also the heterogeneities in its distribution.

PDT of disseminated thoracic malignancies is a potential adjuvant modality for better disease control and improved overall survival in the treatment of malignant pleural mesothelioma (1,2,5,6,9,63) and NSCLC with pleural spread (3). Due to the involvement of the pleural surfaces, treatment with even the most aggressive surgery or radiation therapy compatible with life cannot completely sterilize the region; therefore a local recurrence is inevitable (64-66). PDT can potentially fill this need by providing an adjuvant approach to deliver superficial treatment to large surface areas with a single application (67). It is an evolving application, and thus stands to be benefited by potential insights from pre-clinical studies that model PDT delivery to diffuse thoracic disease. In the present report, we describe an animal model of disseminated thoracic malignancy that is reproducible, well-tolerated, and feasible, together with a technically straightforward procedure to deliver intrathoracic PDT. HPPH-mediated interstitial PDT of the murine thoracic cavity slows progression of disseminated disease with acceptable damage to normal tissue consistent with mechanisms of HPPH action, including vascular disruption and necrotic death. Utilizing MR imaging, it is possible to study PDT effects on tumor burden and distribution, much in the same way that a patient would be evaluated by noninvasive imaging.

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Abbreviations

ALT	alanine aminotransferase
AST	aspartate aminotransferase
BUN	blood urea nitrogen
CPK	creatine phosphokinase
CT	computed tomography
FOV	field of view
HPPH	Photoclor®; 2-[1-hexyloxyethyl]-2-devinyl pyropheophorbide-a
MR	magnetic resonance
MRI	magnetic resonance imaging
mTHPC	Foscan®; m-tetrahydroxyphenylchlorin

NSCLC	non-small cell lung carcinoma
PDT	photodynamic therapy
ROI	region of interest
TE	echo time
TR	repetition time

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Fig. 1.

Growth of human non-small cell lung carcinoma (NSCLC) in the murine thoracic cavity. Representative tumor burden (a) at 12 days after percutaneous intrathoracic injection of H460 cells. Arrows denote millimeter-sized nodules along the pleural surfaces exposed by resection of the lungs and heart. Histopathologic image (b) demonstrating lung carcinoma nodules (arrows) with centralized spontaneous necrosis (*) on a background of murine lung tissue (▲); magnification is 4X. Growth curve (c) of disseminated orthotopic lung carcinoma (collected and weighed at animal necropsy) as a function of time after H460 tumor cell inoculation; data points represent individual mouse tumor burdens with an exponential curve fit and corresponding 95% confidence interval.

Fig. 2. Comparative images of tumor burden obtained at mouse necropsy (left) and by MRI (right). Carcinoma nodule (black *), heart (white *), and right lung (▲) are depicted, respectively. Note that the lung is collapsed at necropsy. Arrows indicate tumor nodules visible by MRI, but hidden by the liver at necropsy.

Fig. 3.

Association between tumor mass and imaged tumor volume. Tumor mass (measured at necropsy) is plotted against tumor volume (with overlying best-fit line and associated 95% confidence interval) assessed from MR images collected along axial (a), coronal (b), and sagittal (c) planes. $r^2 = 0.81, 0.85, \text{ and } 0.88$ for the planes, respectively.

Fig. 4.

Relative change in tumor volume (calculated from MRI slices) between one day before (day -1) and 5 days (day +5) after PDT or control treatment. Histograms represent the mean $\pm$ SD (error bars) intra-animal change in tumor volume for 3-5 animals per group. Left panel displays response in control animals (HPPH-only and light-only); right panel compares PDT with combined control group. * indicates $p < 0.05$, as calculated by the Wilcoxon rank-sum test, when compared to combined control group.

Fig. 5.

Histopathology (10X images) of untreated (a) and PDT-treated (b) murine lung tissue. Untreated lung exhibits normal interstitium with clear alveoli and bronchial spaces; blood vessels are structurally intact. Lung exposed to intrathoracic HPPH-PDT (50 J/cm) contains edema and fibrin deposition of air spaces (single arrow denotes representative alveolus), interstitial congestion (double arrow), perivascular edema (*), and focal vessel disruption with leakage of fibrin (enclosed within diamond) and neutrophils (arrowhead); inset (20X) highlights necrotic foci with pyknotic cell debris (encircled) among infiltrating neutrophils (arrowhead), fibrin (enclosed within diamond), and red blood cells (red).

Table 1Fluence Dependence of Intrathoracic PDT Response^I

Treatment Condition	Tumor Mass (g) Mean $\pm$ SD	Comparison to Untreated p-value
Untreated (n=17)	0.2636 $\pm$ 0.0810	N/A
Light (80 J/cm)-only (n=5)	0.2345 $\pm$ 0.1212	0.6661
HPPH-only (n=11)	0.2928 $\pm$ 0.0805	0.4373
25 J/cm PDT (n=11)	0.2464 $\pm$ 0.1016	0.6891
50 J/cm PDT (n=15)	0.1112 $\pm$ 0.0570	<0.0001
80 J/cm PDT (n=14)	0.1158 $\pm$ 0.0738	<0.0001
150 J/cm PDT (n=6)	0.0829 $\pm$ 0.0447	0.0010
200 J/cm PDT (n=2)	0.1132 $\pm$ 0.0069	0.0236

^I Aggregate tumor burden collected at necropsy 4 days after HPPH-PDT at varying fluences or the indicated controls.

Table 2Spatial Distributions of Tumor Volume Within the Thoracic Cavity¹

	Time Point ²			
	Day -1		Day +5	
Comparison by Location	Mean Relative Tumor Volume (95% CI)	p-value ³	Mean Relative Tumor Volume (95% CI)	p-value ³
Ventral vs. Dorsal	1.73 (1.35, 2.23)	< 0.0001	1.38 (1.19, 1.60)	< 0.0001
Caudal vs. Cranial	1.63 (1.27, 2.10)	0.0002	1.17 (1.01, 1.35)	0.039
Right vs. Left	1.30 (1.01, 1.67)	0.042	1.15 (1.00, 1.32)	0.054

¹Relative difference in tumor volume (from MR imaging) as a function of intrathoracic location, e.g. tumor burden in the ventral half of the thoracic cavity was 1.73-fold greater than burden in the dorsal half on day -1.

²Time points of imaging included one day before and 5 days after PDT (50 J/cm) or control (light-only or drug-only) treatment. Spatial distribution at day +5 was modeled to incorporate PDT effect on overall tumor burden in a location-independent manner as described in the "Methods" section. N=12 animals (4 PDT, 8 control).

³For a test comparing the relative fold change to 1.0.

Table 3

Hematology and Chemistries (Mean Value $\pm$ SD)¹

Treatment Condition	WBC ² (10 ³ /ml)	HgB (g/dl)	PLT (10 ³ /ml)	NEU (% WBC)	LYM (% WBC)	MON (% WBC)	BUN (mg/dl)	Creatinine (mg/dl)	Alk Phos (U/L)	AST (U/L)	ALT (U/L)	CPK (U/L)
Untreated	2.1 $\pm$ 0.8	12.1 $\pm$ 0.8	1003 $\pm$ 84	27.32 $\pm$ 4.27	65.68 $\pm$ 6.54	1.10 $\pm$ 0.44	32 $\pm$ 8	0.37 $\pm$ 0.08	90 $\pm$ 13	115 $\pm$ 81	34 $\pm$ 19	573 $\pm$ 536
HPPH-only	3.4 $\pm$ 1.7	10.8 $\pm$ 3.7	855 $\pm$ 156	27.83 $\pm$ 8.50	67.63 $\pm$ 8.94	0.90 $\pm$ 0.28	30 $\pm$ 8	0.27 $\pm$ 0.12	81 $\pm$ 29	95 $\pm$ 19	20 ³	187 ³
Light-only	4.8 $\pm$ 1.2	12.4 $\pm$ 0.6	918 $\pm$ 88	23.25 $\pm$ 5.54	70.82 $\pm$ 9.55	1.00 $\pm$ 0.43	26 $\pm$ 4	0.30 $\pm$ 0	98 $\pm$ 14	95 $\pm$ 42	24 $\pm$ 6	323 $\pm$ 237
PDT	4.7 $\pm$ 1.8	7.9 $\pm$ 3.4	345 $\pm$ 225 ⁵	86.50 $\pm$ 7.79 ⁵	7.10 $\pm$ 5.37 ⁵	4.03 $\pm$ 0.06 ⁵	39 $\pm$ 16	0.27 $\pm$ 0.12	59 $\pm$ 22	1309 $\pm$ 498 ⁵	296 $\pm$ 72 ⁴	3178 $\pm$ 598 ⁴

¹ Blood collected from tumor-free animals 24 hours after intrathoracic HPPH-PDT to 50 J/cm or the indicated controls; n=3-6 animals except where noted.

² WBC, White blood cell count; HgB, hemoglobin; PLT, platelet count; NEU, neutrophils; LYM, lymphocytes; MON, monocytes; BUN, blood urea nitrogen; Alk Phos, alkaline phosphatase; AST, aspartate aminotransferase; ALT, alanine aminotransferase; CPK, creatine phosphokinase

³ Only 1 sample was available for this condition.

⁴ Two samples were available for these conditions so comparison to untreated is observational (no statistical tests performed).

⁵ p < 0.05 for comparison to untreated samples.