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***In vivo* outcome study of BPD-mediated PDT using a macroscopic singlet oxygen model**

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Abstract

Macroscopic modeling of the apparent reacted singlet oxygen concentration ($[^1\text{O}_2]_{\text{rx}}$) for use with photodynamic therapy (PDT) has been developed and studied for benzoporphyrin derivative monoacid ring A (BPD), a common photosensitizer. The four photophysical parameters (ξ , σ , β , δ) and threshold singlet oxygen dose ($[^1\text{O}_2]_{\text{rx,sh}}$) have been investigated and determined using the RIF model of murine fibrosarcomas and interstitial treatment delivery. These parameters are examined and verified further by monitoring tumor growth post-PDT. BPD was administered at 1 mg/kg, and mice were treated 3 hours later with fluence rates ranging between 75 – 150 mW/cm² and total fluences of 100 – 350 J/cm². Treatment was delivered superficially using a collimated beam. Changes in tumor volume were tracked following treatment. The tumor growth rate was fitted for each treatment condition group and compared using dose metrics including total light dose, PDT dose, and reacted singlet oxygen. Initial data showing the correlation between outcomes and various dose metrics indicate that reacted singlet oxygen serves as a good dosimetric quantity for predicting PDT outcome.

Keywords

photodynamic therapy; PDT; BPD; macroscopic model

1. INTRODUCTION

Photodynamic therapy (PDT) is a cancer treatment modality for cancer and other localized diseases. PDT incorporates light, photosensitizer, and oxygen to create activated singlet oxygen ($^1\text{O}_2$) to kill cells. PDT is uniquely advantageous compared to other treatment modalities as it can be locally delivered, it is non-ionizing, it has fast post-operative recovery, and it has better cosmetic outcome [1]. However, assessing PDT efficacy is difficult due to the lack of accurate dosimetric methods. An explicit singlet oxygen dosimetry model to determine the PDT outcome has been developed and studied [2 – 5]. The four major photochemical parameters in a macroscopic singlet oxygen model have been investigated and determined for the photosensitizer benzoporphyrin derivative monoacid A (BPD) [6]. The previously determined photophysical parameters (ξ , σ , β , δ) and threshold

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singlet oxygen dose ($[^1\text{O}_2]_{\text{rx,sh}}$) were investigated through a tumor growth outcome study to test their validity.

In most clinical settings, PDT dose, i.e., the light dose absorbed by the photosensitizer, is used as the main dosimetric quantity. Under well-oxygenated conditions, PDT dose is a good predictor of treatment outcome. However, both theoretical and experimental mouse studies have shown that in tumors, which are often poorly oxygenated, high fluence rate PDT can create even more hypoxic environments during treatment and result in less effective PDT [2]. In these scenarios, PDT dose is not a good dosimetric quantity. For better measure of PDT outcome, it has been suggested that the production of $^1\text{O}_2$ during PDT based on luminescence signals at 1270 nm should be accounted for [7-9]; however, this is difficult due to its short lifetime (~30-180 ns). Therefore an explicit singlet oxygen dosimetry method can be used for *in vivo* studies by calculating the reacted singlet oxygen ($[^1\text{O}_2]_{\text{rx}}$). This calculation is based on input parameters including photosensitizer characteristics (photophysical parameters), treatment conditions (fluence rate and total fluence), and tumor environment characteristics (initial sensitizer concentration, initial oxygenation state, and optical properties). $[^1\text{O}_2]_{\text{rx}}$ as a dosimetric quantity to predict outcome was investigated in this study. The main focus is that the cumulative reacted singlet oxygen correlates better with PDT outcome than either light or PDT dose alone, as it accounts for PDT-induced oxygen consumption and sensitizer photobleaching.

2. MATERIALS AND METHODS

2.1 Tumor Model

Radioactively induced fibrosarcoma (RIF) cells were cultured and injected subcutaneously at 1×10^7 cells/ml in the right shoulders of 6-8 week old female C3H mice (NCI-Frederick, Frederick, MD). 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. The fur of the tumor region was clipped prior to cell inoculation, and the treatment area was depilated with Nair at least 24 hours before measurements.

2.2 Parameter Determination Studies

Determination of the photophysical parameters were done by a previous study involving partial treatment and determination of the necrosis radius of tumors [2 – 6]. BPD was administered intravenously via the tail at a concentration of 1 mg/kg. Treatment was delivered 3 hours after the injection.

Two catheters were inserted into tumors. One catheter was used to deliver treatment light interstitially using a 1 cm long cylindrically diffusing fiber and 690 nm treatment light. The other catheter was used to insert a detector to measure light fluence inside the tumor as well as obtain a profile to determine the tumor optical properties. A side-firing fiber was also inserted to measure fluorescence excited by 405 nm light both before and after treatment. A number of experiments were performed using different treatment conditions. Necrosis radius

was determined for each tumor by measuring the areas of necrosis on digitally scanned slides of tumor sections stained with hematoxylin and eosin (H & E).

Using the light, sensitizer, and necrosis radius as input parameters, a fitting algorithm was performed in Matlab (to determine the photophysical parameters of BPD. An initial guess of ξ , σ , β , δ , and $[^1\text{O}_2]_{\text{rx,sh}}$ were put into the governing differential equations described in section 2.4. Reacted singlet oxygen profiles, $[^1\text{O}_2]_{\text{rx}}$, were calculated. The fitting routine varied the model parameters globally so that $[^1\text{O}_2]_{\text{rx}}$ at the necrosis radius for each animal (or groups of animals for each treatment condition) is close to the apparent $[^1\text{O}_2]_{\text{rx,th}}$. The objective function of the fitting algorithm is the maximum relative difference between measurements and calculation of the threshold singlet oxygen concentration

$$\text{MAX} \left(\left| 1 - \frac{[^1\text{O}_2]_{\text{rx},i}(r_n)}{[^1\text{O}_2]_{\text{rx,th}}} \right| \right) \quad (1)$$

$[^1\text{O}_2]_{\text{rx},i}(r_n)$ is the computed reacted singlet oxygen at necrosis radius r_n for the i -th mouse (or group of mice).

The photophysical parameters, optical properties, and sensitizer concentrations used for this study are summarized in Table 1.

2.3 Tumor Growth Studies

The tumor growth studies were done with the same type of tumor cells grown on the same location as the parameter determination study with two catheters. However, this time tumors were treated superficially with a collimated beam delivered through an optical fiber with a microlens attachment. Treatment was delivered with a spot size of 1 cm in diameter. Power and fluence rates were measured before every treatment.

Tumors at the time of treatment were at an average volume $\sim 5 - 35 \text{ mm}^3$ (about 7-10 days after the injection of tumor cells). Volumes were monitored every day continuously until the tumor volumes reached $\sim 1000 \text{ mm}^3$. The tumor volume was calculated using the formula $V = \pi/6 \times a^2 \times b$, where a is the width of the tumor and b is the length. To assess and compare the effects of treatment, a tumor growth factor (k) was obtained by fitting the volume to an exponential growth curve of the following form

$$V = Ae^{k \cdot d} \quad (2)$$

Here, d is the number of days after PDT.

A range of total fluences including different source strengths of 50, 75 and 150 mW/cm² and exposure times of 900, 1333, 1667, 1800 and 2700s (total PDT doses of 100, 135 and 250 J/cm²) were used in this study to investigate different levels of treatment and PDT effect. Tumor-bearing mice with no treatment were used as controls.

2.4 Macroscopic Singlet Oxygen Model

A macroscopic singlet oxygen model was used in this study. It has been previously described [2 – 6]. The theory is derived from reaction rate equations for a type II PDT mechanism. The photochemical reactions can be simplified to four coupled differential equations as follows

$$\mu_a \phi - \nabla \cdot \left(\frac{1}{3\mu_s} \nabla \phi \right) = S \quad (3)$$

$$\frac{d[S_0]}{dt} + \left(\xi \sigma \frac{\phi ([S_0] + \delta) [^3O_2]}{[^3O_2] + \beta} \right) [S_0] = 0 \quad (4)$$

$$\frac{d[^1O_2]_{rx}}{dt} - \left(\xi \frac{\phi [S_0] [^3O_2]}{[^3O_2] + \beta} \right) = 0 \quad (5)$$

$$\frac{d[^3O_2]}{dt} + \left(\xi \frac{\phi [S_0]}{[^3O_2] + \beta} \right) [^3O_2] - g \left(1 - \frac{[^3O_2]}{[^3O_2](t=0)} \right) = 0 \quad (6)$$

Here, ϕ is the light fluence rate, S is the source term. The parameters used for this study are fully described in Table 1. The reaction rate constants ($k_{0,1,2,\dots,7}$) are described in Table 2.

For a given value of light fluence rate, ϕ , spatially resolved light fluence rate profiles can be constructed using equation (3), which will then be used in the calculation of the PDT kinetics equations (Eqs. (4)-(6)). The variation of light fluence profiles due to different optical properties can be seen in Figure 2. Most tumors had an average depth of 3 mm, and at this point, the variation in light fluence can be up to ~10%.

The initial sensitizer concentration was determined using data from interstitial measurements as well as validation from an *ex vivo* study, where tumors were excised 3 hours after injection and assayed using a solubilization technique for photosensitizer quantification [10].

2.5 Statistical Analysis

Each treatment condition group for the tumor growth study was independently carried out two times. The tumor growth factor of any two groups is expressed as the mean $\pm$ standard deviation of the measurements. To assess the consistency of the measurements, the tumor volumes in each of the two groups were compared using Kruskal-Wallis tests; 12 – 14 volume values (number of the days post treatment) were considered for each group. Wilcoxon tests were also used to evaluate whether the growth rate of the tumors in each two groups of samples, control and treated mice, are significantly different from each other. Analyses were carried out using SPSS 14.0 software. Statistical significance was defined at $p < 0.05$ level (95% confidence level).

3. RESULTS

The treatment responses were evaluated by the growth rates determined for each treatment condition group. The tumor volumes monitored over time are plotted in Figure 4. These growth rates were fitted by exponential curves of the form described in equation (2), and their growth rates are summarized in Table 3. Some mice were completely cured with the PDT (group number 5). Both mice in this treatment group were found to have no tumor re-growth after 16 days, as seen in Figure 3. Furthermore, the scarring from the PDT effect was shrinking, and hair began to re-grow in the depilated area as well as the PDT treated area. While scarring from PDT was seen for most mice, they also exhibited tumor re-growth post-PDT.

Reacted singlet oxygen was calculated for each PDT treatment condition group and are shown in Figure 3. To evaluate this as a dosimetric quantity, reacted singlet oxygen at a tumor depth of 3 mm ($[^1O_2]_{rx}$ Min) was evaluated as well as the average reacted singlet oxygen from the tumor surface down to the 3mm depth ($[^1O_2]_{rx}$ Mean). These values are summarized in Table 4 along with the tumor growth factors. The correlation between the tumor growth and reacted $[^1O_2]_{rx}$ has been plotted in Figure 5 A. Furthermore, the correlation for the same PDT dose has been highlighted in Figure 5 B, along with the condition that induced complete cure. Here, the total fluence was set to be 135 J/cm² with fluence rates of 50, 75, and 150 mW/cm².

Based on our statistical analysis presented in Table 3, all treatments showed effective control of the tumor growth except for treatment group 1 (75 mW/cm² fluence rate, 1800 s treatment time, 135 J/cm² total fluence). Among the treatment conditions, group number 5 (source strength of 150 mW/cm² and treatment time of 1666.7 s, total light dose of 250 J/cm²) induced complete cure of the tumor. The calculated reacted singlet oxygen concentrations also showed an agreement with the results obtained for the tumor growth rate studies. However, there was a case with more calculated singlet oxygen despite the larger tumor re-growth (group number 4). This group only had one mouse for this condition, so statistical significance is yet to be determined. Further studies with larger numbers of mice per each treatment condition need to be done to generate more statistically significant data.

The results also show that there are very different tumor responses for the same fluence but different fluence rates. This is a clear indicator that PDT dose alone is not a good predictive quantity for PDT treatment outcome. Initial data for the correlations between total light fluence, PDT dose, reacted $[^1O_2]_{rx}$ and tumor growth demonstrate that $[^1O_2]_{rx}$ serves as a better dosimetric quantity for predicting treatment outcome

4. CONCLUSION

A macroscopic model can be used to describe the creation of $[^1O_2]_{rx}$. These equations depend on various input parameters such as the light dose, light source geometry, tissue optical properties, sensitizer concentration, and sensitizer photophysical parameters. The relationship between calculated reacted singlet oxygen using this model and tumor response (tumor growth rate) was investigated in this study. Preliminary data shows that $[^1O_2]_{rx}$ is a

better metric for predicting outcome than PDT dose or light dose alone. Further studies will need to be done to validate these results as well as to establish a threshold $[^1\text{O}_2]_{\text{rx}}$ for this type of treatment.

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Figure 1.

(A) Experimental set-up for superficial PDT. Two mice were treated at a time on a heated water pad under anesthesia. (B) Multi-fiber spectroscopic probe being used to measure diffuse reflectance and fluorescence

Figure 2.

Fluence variation due to optical properties. The ratio between fluence at a tissue depth of d and the fluence at the surface ($d = 0$) is plotted against tissue depth, d . Various optical properties seen for mice from previous studies were plotted. At 3 mm (the average tumor depth for this study), the maximum variation in light fluence is ~10%.

Figure 3. Tumor images for a mouse in group number 5 with complete cure. (A) PDT effect (scarring) can be seen immediately 1 day after treatment. (B) No tumor regrowth was seen after 16 days and scarring began to heal.

Figure 4.
Tumor growth volume monitored over time and fit exponentially

Figure 5. Growth rate versus mean singlet reacted singlet oxygen for (A) all data and (B) all data with the same PDT dose (total light fluence).

Table 1

Photochemical parameters, optical properties, and sensitizer concentrations for BPD studies

Parameter	Definition	Value
ξ (cm ² s ⁻¹ mW ⁻¹)	Specific oxygen consumption rate $\xi = S \left(\frac{k_5}{k_5 + k_3} \right) \frac{e}{h\nu} \frac{k_7[A]/k_6}{k_7[A]/k_6 + 1}$	$(51 \pm 15) \times 10^{-3}$
σ (μM ⁻¹)	Specific photobleaching ratio $k_1/k_7[A]$	$(1.7 \pm 0.3) \times 10^{-5}$
β (μM)	Oxygen quenching threshold concentration k_4/k_2	11.9
δ (μM)	Low concentration correction	33
g (μM/s)	Macroscopic oxygen maximum perfusion rate	2.4 ± 0.7
$[^1O_2]_{rx,sh}$ (mM)	Singlet oxygen threshold dose	0.72 ± 0.21
μ_a (cm ⁻¹)	Absorption coefficient	0.66 ± 0.15
μ_s' (cm ⁻¹)	Reduced scattering coefficient	9.88 ± 2
$[S_0]$ (μM)	Initial photosensitizer concentration	0.60 ± 0.25

Table 2

Reaction rate constants in a type II PDT process

Symbol	Definition
k_0	Photon absorption rate of photosensitizer per photosensitizer concentration
k_1	Bimolecular rate for $^1\text{O}_2$ reaction with ground-state photosensitizer
k_2	Bimolecular rate of triplet photosensitizer quenching by $^3\text{O}_2$
k_3	Decay rate of first excited singlet state photosensitizer to ground state photosensitizer
k_4	Rate of monomolecular decay of the photosensitizer triplet state
k_5	Decay rate of first excited state photosensitizer to triplet state photosensitizer
k_6	$^1\text{O}_2$ to $^3\text{O}_2$ decay rate
k_7	Bimolecular rate of reaction of $^1\text{O}_2$ with biological substrate [A]
S	Fraction of triplet-state photosensitizer; $^3\text{O}_2$ reactions to produce $^1\text{O}_2$

Table 3

Kruskal-Wallis and Wilcoxon statistical analyses

Groups for the analyses	<i>p</i> ^b	Pairs for the comparisons	<i>p</i>
75 mW/cm ² , 1800 s, 135 J/cm ²	0.45	75 mW/cm ² , 1800 s, 135 J/cm ² & Control	0.67
75 mW/cm ² , 1333 s, 100 J/cm ²	0.48	75 mW/cm ² , 1333 s, 100 J/cm ² & Control	0.002
150 mW/cm ² , 2333 s, 350 J/cm ²	0.98	150 mW/cm ² , 2333 s, 350 J/cm ² & Control	0.013
150 mW/cm ² , 1667 s, 250 J/cm ²	-	150 mW/cm ² , 1667 s, 250 J/cm ² & Control	-
150 mW/cm ² , 900 s, 135 J/cm ²	0.19	150 mW/cm ² , 900 s, 135 J/cm ² & Control	0.32
Control ^a	0.34		

^aThe mice with no PS and no treatment.

^b*p* values obtained by Kruskal-Wallis and Wilcoxon tests are generated by comparing the values of each two groups. In all cases $p < 0.001$ showing a statistically significant difference with 95% confidence level.

Table 4

Tumor growth factors and calculated amounts of singlet oxygen for each PDT treatment condition. R^2 values are for the exponential fits for each growth curve.

#	Source Strength (mW/cm ²)	Time (s)	Fluence (J/cm ²)	Growth Rate, k (days ⁻¹)	R^2	[¹ O ₂]rx Min (mM)	[¹ O ₂]rx Mean (mM)
1	75	1800	135	0.3381 ± 0.05	0.9962	0.7989	0.7437
2	75	1333	100	0.3360 ± 0.02	0.9968	0.9163	0.831
3	50	2700	135	0.2392	0.9821	0.9008	0.8393
4	150	2333.3	350	0.2523 ± 0.00	0.9817	1.5837	1.5664
5	150	1666.7	250	0	1	1.1574	1.1268
6	150	900	135	0.3335 ± 0.06	0.9979	0.5955	0.5535
7	Control			0.4025 ± 0.01	0.9967	0	0