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Article

EFFECTS OF LIGHT DOSIMETRY IN PHOTOBLEACHING OF THE PHOTOSENSITIZER (PS) AND CYANINE DYE (CD) MOIETIES IN PS-CD CONJUDATES AND ITS CORRELATION WITH PHOTODYNAMIC THERAPY (PDT) EFFICACY

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Abstract: Photodynamic therapy (PDT) of cancer is dependent on three primary components: photosensitizer (PS), light, and oxygen. Because these components are interdependent and vary during the dynamic process of PDT, assessing PDT efficacy may not be trivial. Therefore, it has become necessary to develop pre-treatment planning, on-line monitoring and dosimetry strategies during PDT, which become more critical for two or more chromophore systems, e.g. PS-CD conjugates developed in our laboratory for fluorescence-imaging and PDT of cancer.

In this study, we observed a significant impact of variable light dosimetry; (i) high light fluence and fluence rate (light dose: 135 J/cm^2, fluence rate: 75 mW/cm^2) and (ii) low light fluence and fluence rate (128 J/cm^2 and 14 mW/cm^2 and 128 J/cm^2 and 7 mW/cm^2) in photobleaching of the individual chromophores and their long-term tumor response. The fluorescence at the near-infrared (NIR) region of the PS-NIR fluorophore conjugate was assessed intermittently via fluorescence imaging. The loss of fluorescence, photobleaching, caused by singlet oxygen from the PS was mapped continuously during PDT. The tumor responses (BALB/c mice bearing Colon26 tumors) were assessed after PDT by measuring tumor sizes daily. Our results showed distinctive photobleaching kinetics rates between the PS and CD. Interestingly, compared to higher light fluence, the tumors exposed at low light fluence showed reduced photobleaching and enhanced long-term PDT efficacy. The presence of NIR fluorophore in PS-CD conjugates provides an opportunity of fluorescence imaging and monitoring the photobleaching rate of the CD moiety for large and deeply seated tumors and assessing PDT tumor response in real-time.

Keywords: Photodynamic therapy, photobleaching, photosensitizers, fluorescence imaging.

1. Introduction

PDT was initially developed for the local destruction of solid tumors [1, 2] and is currently being used worldwide in the treatment of several tumors including skin basal cell carcinoma (BCC) [3], lung [4-6], esophagus [7-11], bladder, head and neck [4,6,12], brain [13-17], ocular melanoma, ovarian, prostate [8-20], renal cell, cervix, pancreas and bone [21]. It is also being used for a plethora of additional indications such as, dysplasia, papillomas, rheumatoid arthritis, age related macular degeneration, actinic keratosis, cosmesis, psoriasis, endometrial ablation, localized infection (bacterial and fungal) and prophylaxis of arterial restenosis. Considering that the use of PDT has been approved for many diseases, it is still not being practiced in mainstream oncology. The partial reason for this is that the treatment outcome cannot be foreseen clearly, as the therapy related dosimetry based on measured or calculated physical values is not yet optimized.

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PDT is known to be dependent on three primary components: Photosensitizer (PS), light, and oxygen in order to deliver an effective dose[1]. Therefore, it is necessary to establish an understanding of the basic physical and biophysical interactions of these three essential components to maximize PDT output. Over the past two decades, much work has been done to optimize these components, but their dynamic nature and complex interdependency lead to complexity. Therefore, to understand the PDT dosimetry, which is intended to quantify a therapeutic outcome, is of importance to provide reliable tool for controlled enhancement of the PDT outcome.

Currently most of the photosensitizers (PSs) used in PDT elicit significant damage to cancer cells through singlet oxygen mediated pathways [1]. The standard approach in clinical PDT is to use the prescribed treatments that involve using fixed amounts of photosensitizer (per unit body weight), incident light fluence (J/cm^2) and fluence rates (total energy delivered within a specific drug-light time interval (mW/cm^2)) [22] to treat each patient. However, this sometimes leads to incomplete or unpredictable responses in patient groups, which may be due to differences in individual physiological effects [22,23]. These heterogeneous factors include local tissue optical properties, tumor oxygenation and accumulated photosensitizer dose, which can be very different for each patient and tumor. These factors also may change differently during PDT. For example, photobleaching of the PS may reduce singlet oxygen production which may cause ground-state oxygen to be depleted if the reperfusion capacity of the tissue is exceeded by the immediate photochemical reaction [24]. Therefore, it has become necessary to develop pretreatment planning, on-line monitoring and dosimetry strategies during PDT [1].

Optimization of clinical dosimetry methods can follow one of three paths as described previously (see for example McIlroy *et al* [24], *Wilson et al* [25] and Zhu [26]) and can be classified as direct [26], explicit or implicit dosimetry [22,25]. Direct dosimetry involves the measurement of singlet oxygen itself, either through emission of its phosphorescence or through singlet oxygen sensitive chromophores [26]. Explicit dosimetry employs techniques and instrumentation to measure the three essential components of photodynamic therapy (light, photosensitizer (PS), and oxygen) individually and independently in the tissue [22,25]. A predictive model of the photobiological effect of these three components is required to combine the measurements into a dose metric [22,23,25]. Significant progress has been made in regards to the application of explicit dosimetry but there are still limitations [25]. Implicit dosimetry seeks to avoid measuring the light, PS and oxygen independently by eliciting the use of a single parameter that incorporates two or more of the essential components into a single metric in order to predict the biological damage [1,22,25,27]. This is chiefly accomplished by monitoring the PS photobleaching during irradiation by utilizing the fluorescence properties of the PS [22,25].

We are engaged in the exploration of PDT dosimetry strategies by employing the implicit dosimetry approach. In our strategy, we aim to investigate the utility of the photosensitizer-near infrared fluorophores conjugate (PS-NIRF) as fluorescence probes and markers for PDT light dosimetry. We hypothesize that the photobleaching characteristics of the fluorophores when subjected to variable light fluence and fluence rates will provide a metric to optimize the light dosimetry and PDT response.

If singlet oxygen is the main cytoxic agent and the main cause of photobleaching, monitoring the photobleaching could provide a quantifiable measure of the singlet oxygen production. Therefore, we monitored the photobleaching of the photosensitizer and the fluorophore within several bifunctional photosensitizer-fluorophore conjugates using real time *in vivo* fluorescence imaging. In our initial attempt, the fluorescence of the NIR fluorophore portion of the conjugate was measured intermittently throughout the PDT treatment. First, *in vitro* photobleaching experiments were performed as a model of the *in vivo* systems to see if they could predict the *in vivo* response. Then, we imaged mice before and after PDT treatment at three different light doses, after exciting

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the PS or the NIRF portion of the conjugate individually, and investigated potential direct correlation between photobleaching and PDT efficacy.

2. Results and Discussion

The degradation of the conjugates was observed via UV-Vis spectroscopy *in vitro*. Although these solutions of conjugates were made up as 5 μM solutions in 17% Bovine Calf Serum (BCS) it was understood that the intent was not to reproduce exact solvent conditions used clinically but to show whether drug aggregation or binding with serum affected the photobleaching of the chromophores. However, in our *in vivo* photobleaching experiments the degradation of the NIR fluorophore (CD) portion of the PS-CD conjugates was observed. In later experiments, we observed the degradation of both PS and CD via fluorescence quenching.

2.1. Mechanism of Photo-induced Bleaching

Using the NIR CD as a guide, **Figure 1**, illustrates the mechanism of photo-degradation caused by the contribution of molecular oxygen and light. Interaction of singlet oxygen with the chromophores constitutes the major pathway of photodecomposition. When the PS (HPPH) portion of the PS-NIR fluorophore (CD) conjugate absorbs light it undergoes intersystem crossing from an excited singlet state to an excited triplet state where it interacts with endogenous ground state triplet oxygen to generate the destructive singlet oxygen species. In the illustration below, the singlet oxygen generated subsequently attacks the polymethine chain of cypate resulting in fragmentation of the CD moiety as shown in **Figure 1**.

The photoproduct obtained after photo-induced bleaching is due to oxidation of the C′- C2 or C7′- C2′′ bond on the polymethine chain. Singlet oxygen is directed towards the polymethine chain at the C′- C2 or C7′- C2′′ bond due the electro-positivity of the 2 and 2″ carbon versus the electron rich position 1′ or 7′ of the cationic chromophore [28]. This degradation generates the corresponding carbonyl photoproducts [28].

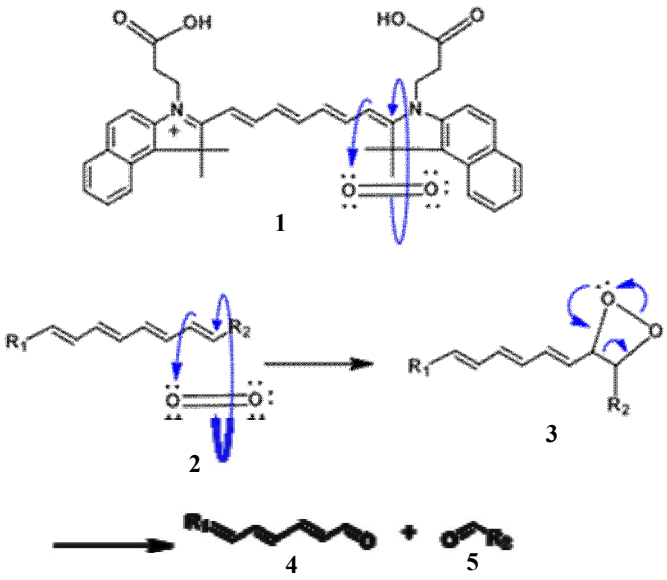

Figure 1: Mechanism of the photobleaching of Cypate by singlet oxygen. This illustrates the photobleaching that occurs in vitro and *in vivo* following absorbance of light in NIR region of the spectrum.

2.2. *In Vitro* Photobleaching of HPPH Cyanine Dye and CD Conjugates

The conjugates; HPPH-CD (**8**), HPPH2CD (**9**), HPPH-Cypate (**10**) and HPPH2-Cypate (**11**) shown in **Figure 2** were prepared by following our own methodology were formulated in 17% Bovine Calf Serum/PBS and were used equimolar concentrations (5 μM) for the studies presented herein [29, 30]. The absorption spectra were measured in 1 cm-quartz cuvettes following irradiation at 665 nm at various time points until there was complete degradation of the NIRF portion of the conjugate, **Figure 3**. The photobleaching rates were different, and were in the following order **9** > **10** > **8** > **11**.

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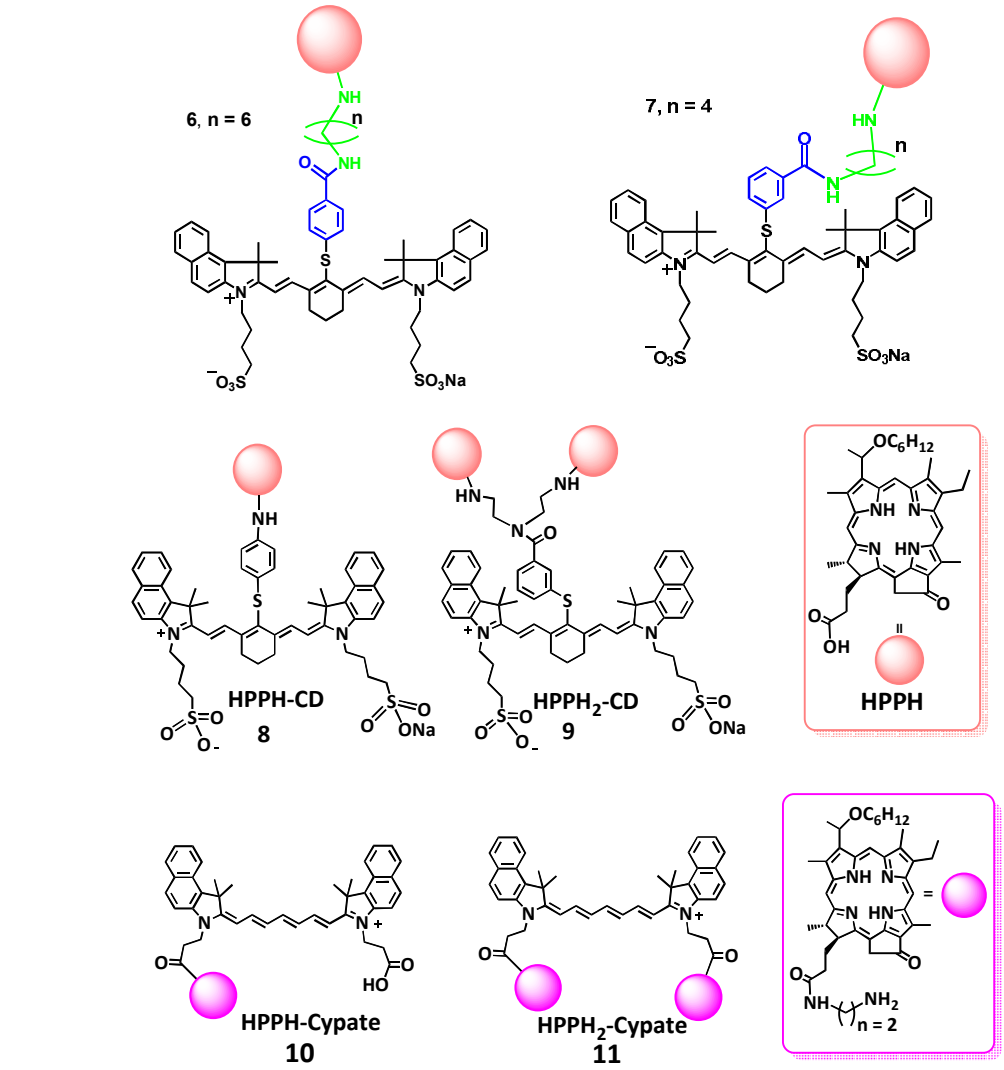

Figure 2: Structures of the conjugates **6-11** used for the study

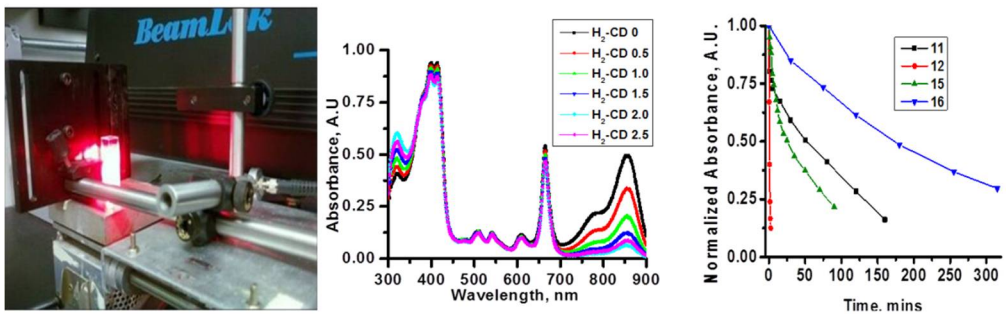

Figure 3: Photobleaching of the conjugates as 5 uM solutions in 17% BCS in PBS. HPPH-CD (**8**), HPPH2CD (**9**), HPPH-Cypate (**10**) and HPPH2-Cypate (**11**) were photobleached *in vitro* in the order of **9 > 10 > 8 > 11**.

2.3. *In Vivo* Photo-induced bleaching kinetics

BALB/c mice (3/group) were inoculated with compounds, **8, 9, 10** and **11** and monitored during PDT for 30 minutes at various time intervals. The tumors were irradiated at a wavelength 665 nm using a total light dose of 135 J/cm² and fluence rate of 75 mW/ cm², respectively. Concurrently, the fluorescence kinetics were monitored by illuminating at 785 nm and measuring the fluorescence

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with a 830 long pass filter. Figure 3 shows images of the photo-induced bleaching kinetics of the four conjugates **8, 9, 10** and **11** at different time points during treatment. The mice injected with HPPH-Cypate (**10**) were irradiated at 661 nm. PDT efficacy of HPPH-CD (**8**), HPPH2CD (**9**), HPPH-Cypate (**10**) and HPPH2Cypate (**11**) was also assessed after the photo-induced bleaching experiment, as shown in Figure 4. We observed that there were no PDT cures upon assessment of the tumor response (**Figures 4 & 5**). All mice were sacrificed when the tumor sizes grew to a volume of 400 mm^3. The dismal tumor response could be due to changes in tumor oxygenation induced by high fluence rates [31]. High fluence rates such as that used in this experiment (135 J/cm^2 and 75 mW/cm^2) can cause the depletion of molecular oxygen during the process of singlet oxygen generation, which can exceed the rate at which it can be resupplied by diffusion from the vasculature [31].

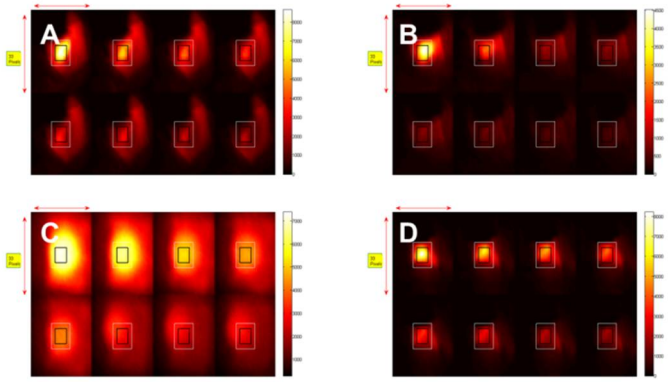

Figure 4: Photobleaching of (A) HPPH-CD (**8**), (B) HPPH2CD (**9**), (C) HPPH-Cypate (**10**) and (D) HPPH2Cypate (**11**) All conjugates were irradiated at 665 nm, except in the case 15 (661 nm) and treated at a fluence and fluence rate of 135 J/cm^2 and 75 mW/cm^2. Concurrently fluorescence images were taken at various treatment times up to 30 minutes, the total treatment time

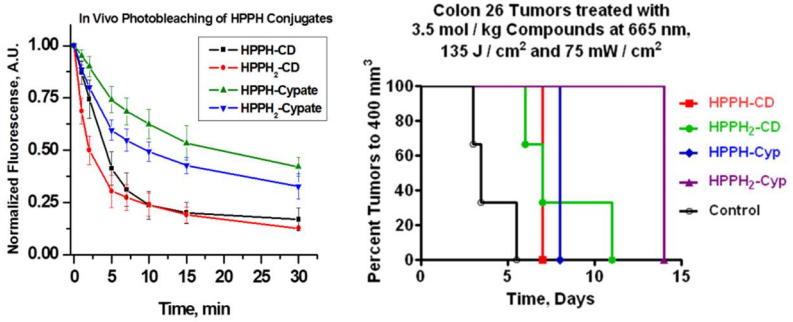

Figure 5: *In vivo* phot bleaching of the conjugates HPPH-CD (**8**), HPPH2CD (**9**), HPPH-Cypate (**10**) and HPPH2-Cypate (**11**) occurred in the order of **9** > **8** > **11** > **10**. There were no PDT cures upon assessment of the tumor response. All mice were sacrificed when they grew to a volume of 400 mm^3.

It is believed that such occurrences would lead to PDT self-limiting hypoxic conditions [31] whereby the tumor and surrounding regions becomes deprived of oxygen. Therefore we adapted the following experiments with the low-fluence rates.

2.4. *In Vivo* Photobleaching Before and After Low-fuence rate PDT Treatments

It has been demonstrated that there was a direct relationship between the tumor response and the level of oxygen within the tumor tissue during PDT [31-38]. Additionally, it has also been demonstrated that using HPPH as a PS and exposing the tumor at low light fluence and fluence rate of 128 J/cm^2 and 14 mW/cm^2 showed better tumor response rates with cures up 90 days after PDT [31,33]. Another observation made during these experiments was that the tumor response for the fluence of 128 J/cm^2 increased as the fluence rate decreased further [31,33]. Therefore, to understand the impact of these light treatment parameters in PDT efficacy of the PS-CD conjugates, the photobleaching experiments were conducted at various fluence and fluence rates of 135 J/cm^2 and 75 mW/cm^2; 128 J/cm^2 and 14 mW/cm^2 and 128 J/cm^2 and 7 mW/cm^2

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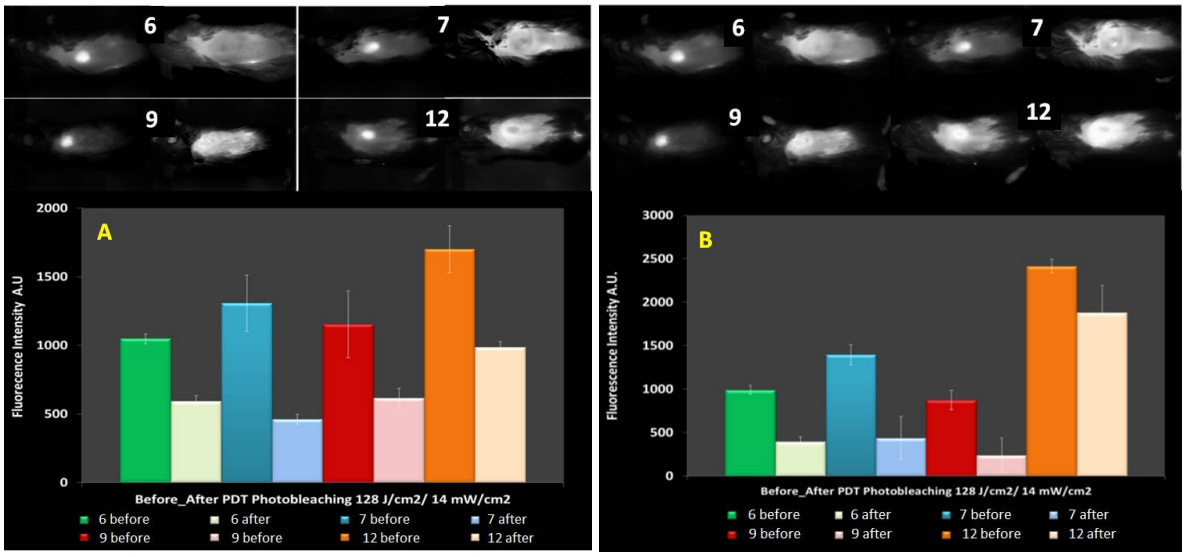

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Figure 6: (A) Photobleaching of the HPPH portion of PS-CD conjugates, and **(B)** a comparative photobleaching of the CD moiety in HPPH-CD conjugates **6, 7, 9 and 12** at the light dose of 128 J/cm² and 14 mW/cm²

In these experiments, the fluorescence was observed before and after PDT treatment at the fluence and fluence rates mentioned above. The compounds chosen for this study were conjugates **6**, **7**, 9 **(Figure 2)** and **12** (**Figure 7**) on the basis of their significant *in vitro* and *in vivo* PDT responses in mice bearing Colon-26 and U87 tumor models [29,30].

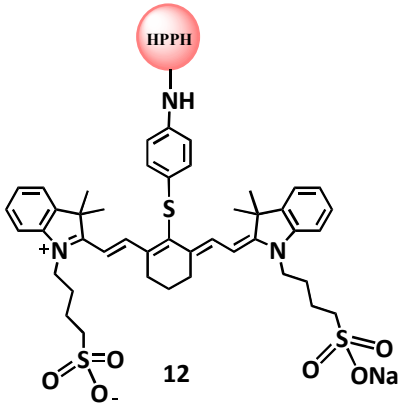

Figure 7: Structure of HPPH-CD conjugate **12**

Compounds			**Fluorescence**				**Photobleached 128 J/cm² 14mW/cm²**		**% Tumor Cured 128 J/cm² 14mW/cm²**
Drug #	PS	Dose (umol /kg)	Pre PDT HPPH	Pre PDT CD	Post PDT HPPH	Post PDT CD	% PBLCH HPPH	% PBLCH CD	PDT ONLY
6	LLP	1.5	1048	989	592	397	43	60	80
7	MLM	1.5	1308	1396	460	439	65	69	80
9	H2CH	1.5	1154	874	616	239	47	73	80
12	H783NH2	1.5	1701	2413	986	1881	42	22	40

Table 1: Summary of *in vivo* photobleaching of HPPH moiety in HPPH-CD conjugates **6, 7, 9** and **12** at 128 J/cm² and **14** mW/cm²

The photobleaching data obtained from these experiments were used in conjunction with the molecular modeling of the compounds to infer possible optimization of PDT light dosimetry since it has been reported that it is not simple to predict the photobiological outcome from *in vivo*

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photobleaching data alone, because of the complex dependence on oxygenation and micro-environment factors [22].

The fluorescence intensities of HPPH and the CD were also compared prior to light irradiation and at the end of treatment with light dose of 128 J/cm^2 and 14 mW/cm^2. After combining the data obtained from the three mice in each group it was found that the HPPH (PS) portion of compounds **6, 7, 9** and **12** photobleached by 43%, 65%, 47% and 42% respectively; whereas, the CD portion underwent photoleaching by 60%, 69%, 73%, and 22% respectively.

Compounds			**Fluorescence**				**Photobleached 48 J/cm^2 7mW/cm^2**		**% Tumor Cured 48 J/cm^2 7mW/cm^2**
Drug#	PS	Dose (µmol/kg)	Pre PDT HPPH	Pre PDT CD	Post PDT HPPH	Post PDT CD	% PBLCH HPPH	% PBLH CD	PDT ONLY
6	LLP	1.5	1060	976	825	492	22	50	ND
7	MLM	1.5	1485	1319	1170	1019	21	23	ND
9	H2CH	1.5	986	809	808	459	18	43	ND
12	H783NH2	1.5	1428	2538	1238	1783	13	30	ND

Table 2: Summary of the photobleaching results with conjugates **6, 7, 9** and **12** at a light dose of 48 J/cm^2 and 7mW/cm^2. The PDT activity of the conjugates at a light dose of 48J/cm^2 , 7mW/cm^2 was not determined (ND).

Under similar treatment parameters, the photobleaching rates of the CD portion of the conjugate in **6, 7,** and **9** were observed in the range of 60 – 73% when irradiated at 128 J/cm^2 and 14mW/cm^2 On the other hand, the PDT response was 40% for conjugate **9** with only 20% photobleaching observed for the CD (**Table 1**). These results suggest that there is a direct correlation between the rate of photobleaching of the CD and the tumor response; more the photoleaching of the CD in HPPH-CD conjugates, the higher was the tumor response. It was difficult to infer a similar conclusion in the case of HPPH photobleaching with respect to tumor response, since the photobleaching rates were quite similar for all the cases except compound **6**. Summary of photobleaching rates of **6, 7, 9** and **12** at 128 J/cm^2 and 14 mW/cm^2 are shown in Tables 1 & 2.

There was no PDT response when a fluence and fluence rate of 128 J/cm^2 and 7 mW/cm^2 was used. This can be explained by the threshold dose beyond which the repair of sub-lethal damage overrides the advantages of low fluence rate [31,39,40]. However, in the experiment where fluence rates of 135 J/cm^2 and 75mW/cm^2 (**Table 2**) were used, the photo-induced bleaching of the CD portion of the conjugate was between 16% and 52%, and there was a tumor response of 66% and 33% for compounds **9** and **12,** respectively. The degree of photobleaching of the HPPH moiety also correlated well with the NIRF moiety. Therefore, the less photobleached the HPPH or NIRF at 135 J/cm^2 and 75 mW/cm^2 the better was the response. This could also be due to with the amount accumulated conjugate (PS) within the tumor before PDT light illumination. It has been shown that the combination of fluence and fluence rate can lead to anoxic conditions within the first few minutes of illumination [31,33]. Therefore, it can be deduced that if the amount of photosensitizer accumulated within the tumor prior to PDT is high this would help to circumvent the observance of anoxic conditions

Compounds			Fluorescence				Photobleached 135 J/cm² 75mW/cm²		% Tumor Cured 135 J/cm² 75mW/cm²	
Drug #	PS	Dose (umol/ kg)	Pre PDT HPPH	Pre PDT CD	Post PDT HPPH	Post PDT CD	% PBLCH HPPH	% PBLCH CD	% Tumor cure	
6	LLP	1.5	1136	1025	670	416	41	60	0	
7	MLM	1.5	1380	1319	655	560	53	58	0	
9	H2CH	1.5	996	904	683	430	31	52	33	
12	H783NH2	1.5	1392	2543	1390	2143	8	16	66	

Table 3: Summary of the photobleaching of conjugates **6, 7, 9** and **12** at 135 J/cm² and 75 mW/cm²

3. Conclusion

The data acquired from the *in vitro* photo-induced bleaching of conjugates showed that NIR fluorophore CD of the conjugates photobleached at a much faster rate than HPPH and the rate of bleaching was in the order of **9 > 10 > 8 > 11**, which correlated well with their *in vivo* PDT responses when irradiated at 128 J/cm² and 14 mW/cm², using a drug dose of 1.5 umol/kg. Conjugate **12** produced 80% tumor response, whereas compounds **10** and **11** did not yield any long-term cure. The *in vivo* photobleaching of HPPH and CD in conjugates before and after PDT suggests that measuring the rate of the photobleaching of CD could be a useful tool to optimize the PDT light dosimetry. We hypothesize that the determining the rate of photobleaching of CD in PS-CD conjugates may help to measure indirectly the amount of singlet oxygen generation during photodynamic therapy treatment at variable light fluence and fluence rates [1, 22]. These studies are currently underway.

According to Wilson et al the use of photobleaching as a dose metric is based on the fact that the photosensitizer or NIR fluorophore will be degraded directly or indirectly by singlet oxygen as it goes through each photo-activation cycle [41]. An enhanced level of photo-activation is directly proportional to enhanced photobleaching. As a result, a hypothesis is proposed that greater photobleaching represents greater singlet oxygen production and hence an enhanced photodynamic efficacy [41]. However, this assumption/hypothesis may lead to several issues such as: (i) If the fluorescence of the PS is used for the measurements without knowing the absolute initial concentration of the PS, it could be difficult to determine the absolute number of PS molecules photobleached per unit volume during PDT [39]. It has been shown, however, that this requirement can be fulfilled by quantitative fluorescence imaging methods that can quantify absolute PS concentrations (ii) Molecular changes of the PS within tissue may result in changes to the fluorescence without any changes in PDT response [41]; (iii) Photobleaching rate itself may not be a sole indicator for PDT dosimetry/response [41], since it has been shown that singlet oxygen generation is dependent on the tissue microenvironment [4, 41]. The micro/local-distribution of the PS will be difficult to quantify because noninvasive or noninvasive fluorescence measurements are limited, and can only provide a measure of average/bulk value of the PS concentration.

Author Contributions:

Nadine James: Synthesis of the conjugates and biological evaluation (in vitro, in vivo) evaluation of the compounds. Nadine also prepared the first draft of the manuscript. Ravindra R. Cheruku: Synthesized some of

the PS-CD conjugates required to confirm the photophysical properties. Joseph Missert: Isolated the starting material from *Spirulina pacifica*, which mainly contains chlorophyll-a. Ulas Sunar: Designed the *in vivo* photobleaching experiments, helped in analyzing the data and also providing helpful comments on the manuscript. Ravindra K Pandey: Overall supervision of the project including the design of the conjugates studied in this project, providing financial assistance from the NIH funded grant in which he is the PI, and refining the manuscript before submission.

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Conflicts of Interest: The authors declare no conflict of interest.

References

1. Jarvi Mark, T., Niedre Mark, J., Patterson Michael, S. & Wilson Brian, C. Singlet oxygen luminescence dosimetry (SOLD) for photodynamic therapy: current status, challenges and future prospects. *Photochem Photobiol* **2006**, *82*, 1198-1210.
2. Wilson, Brian C. & Patterson, Michael S. The physics, biophysics and technology of photodynamic therapy. *Physics in Medicine & Biology* **2008**, *53*, R61-R109.
3. Fien, Sari M. & Oseroff, Allan R. Photodynamic therapy for non-melanoma skin cancer. *J. Natl. Compr. Cancer Network* **2007**, *5*, 531-540.
4 Biel, M. A. Photodynamic therapy and the treatment of head and neck cancers. *Journal of clinical laser medicine & surgery* **1996**, *14*, 239-244.
5 Friedberg, Joseph S., Mick, Rosemarie, Stevenson, James P., Zhu, Timothy, Busch, Theresa M., Shin, Daniel, Smith, Debbie, Culligan, Melissa, Dimofte, Andreea, Glatstein, Eli & Hahn, Stephen M. Phase II trial of pleural photodynamic therapy and surgery for patients with non-small-cell lung cancer with pleural spread. *J. Clin. Oncol.* **2004**, *22*, 2192-2201.
6 Grant, W. E., Speight, P. M., Hopper, C. & Bown, S. G. Photodynamic therapy: an effective, but non-selective treatment for superficial cancers of the oral cavity. *International journal of cancer. Journal international du cancer* **1997**, *71*, 937-942.
7 Ackroyd, R., Brown, N. J., Davis, M. F., Stephenson, T. J., Marcus, S. L., Stoddard, C. J., Johnson, A. G. & Reed, M. W. R. Photodynamic therapy for dysplastic Barrett's esophagus: A prospective, double blind, randomized, *placebo controlled trial. Gut* **2000**, *47*, 612-617.
8 Overholt, B. F. & Panjehpour, M. Photodynamic therapy for Barrett's esophagus: clinical update. *Am J Gastroenterol* **1996**, *91*, 1719-1723.
9 Overholt, B. F., Panjehpour, M. & Ayres, M. Photodynamic therapy for Barrett's esophagus: cardiac effects. *Lasers Surg Med* **1997**, *21*, 317-320.
10 Overholt, B. F., Panjehpour, M. & Haydek, J. M. Photodynamic therapy for Barrett's esophagus: follow-up in 100 patients. *Gastrointestinal endoscopy* **1999**, *49*, 1-7.
11 Overholt, Bergein F., Wang, Kenneth K., Burdick, J. Steven, Lightdale, Charles J., Kimmey, Michael, Nava, Hector R., Sivak, Michael V., Jr., Nishioka, Norman, Barr, Hugh, Marcon, Norman, Pedrosa, Marcos, Bronner, Mary P., Grace, Michael & Depot, Michelle. Five-year efficacy and safety of photodynamic therapy with Photofrin in Barrett's high-grade dysplasia. *Gastrointestinal endoscopy* **2007**, *66*, 460-468.
12 Biel, M. Advances in photodynamic therapy for the treatment of head and neck cancers. *Lasers Surg Med* **2006**, *38*, 349-355.
13 Bogaards, Arjen, Varma, Abhay, Zhang, Kai, Zach, David, Bisland, Stuart K., Moriyama, Eduardo H., Lilge, Lothar, Muller, Paul J. & Wilson, Brian C. Fluorescence image-guided brain tumor resection with adjuvant metronomic photodynamic therapy: pre-clinical model and technology development. *Photochem. Photobiol. Sci.* **2005**, *4*, 438-442.
14 Rosenthal, Mark A., Kavar, Bhadu, Hill, John S., Morgan, Denis J., Nation, Roger L., Stylli, Stanley S., Basser, Russell L., Uren, Shannon, Geldard, Howard, Green, Michael D., Kahl, Stephen B. & Kaye, Andrew H. Phase I and pharmacokinetic study of photodynamic therapy for high-grade gliomas using a novel boronated porphyrin. *J. Clin. Oncol.* **2001**, *19*, 519-524.
15 Stylli, Stanley S., Howes, Megan, MacGregor, Lachlan, Rajendra, Priya & Kaye, Andrew H. Photodynamic therapy of brain tumours: evaluation of porphyrin uptake versus clinical outcome. *J. Clin. Neurosci.* **2004**, *11*, 584-596.

16 Stylli, Stanley S. & Kaye, Andrew H. Photodynamic therapy of cerebral glioma - A review Part I - A biological basis. *J. Clin. Neurosci*. **2006**, *13*, 615-625.

17 Stylli, Stanley S., Kaye, Andrew H., MacGregor, Lachlan, Howes, Megan & Rajendra, Priya. Photodynamic therapy of high grade glioma - long term survival. *J. Clin. Neurosci*. **2005**, *12*, 389-398.

18 Stripp, Diana C. H., Mick, Rosemarie, Zhu, Timothy C., Whittington, Richard, Smith, Debbie, Dimofte, Andreea, Finlay, Jarod C., Miles, Jeremy, Busch, Theresa M., Shin, Daniel, Kachur, Alex, Tochner, Zelig A., Malkowicz, S. Bruce, Glatstein, Eli & Hahn, Stephen M. Phase I trial of motexafin-lutetium-mediated interstitial photodynamic therapy in patients with locally recurrent prostate cancer. *Proc. SPIE-Int. Soc. Opt. Eng*. **2004**, *5315*, 88-99.

19 Weersink, Robert A., Wilson, Brian C., Bogaards, Arjen, Gertner, Mark R., Davidson, Sean R. H., Haider, Masoom A., Elhilali, Mostafa & Trachtenberg, John. Vascular-targeted photodynamic of prostate cancer phase with Tookad for recurrent prostate cancer following radiation therapy: Initial clinical studies. *Proc. SPIE-Int. Soc. Opt. Eng*. **2007**, 6424, 64241E/64241-64241E/64210.

20 Weersink Robert, A., Bogaards, Arjen, Gertner, Mark, Davidson Sean, R. H., Zhang, Kai, Netchev, George, Trachtenberg, John & Wilson Brian, C. Techniques for delivery and monitoring of TOOKAD (WST09)-mediated photodynamic therapy of the prostate: clinical experience and practicalities. *Journal of photochemistry and photobiology. B, Biology* **2005**, *79*, 211-222.

21 Miyagi, Kiyoko, Sampson, Reynee W., Sieber-Blum, Maya & Sieber, Fritz. Crystal violet combined with Merocyanine 540 for the ex vivo purging of hematopoietic stem cell grafts. Journal of Photochemistry and *Photobiology, B: Biology* **2003**, *70*, 133-144.

22 McIlroy, Brian W., Mann, Thomas S., Dysart, Jonathan S. & Wilson, Brian C. The effects of oxygenation and photosensitizer substrate binding on the use of fluorescence photobleaching as a dose metric for photodynamic therapy. *Vibrational Spectroscopy* **2002**, *28*, 25-35.

23 Ascencio, Manuel, Collinet, Pierre, Farine, M. O. & Mordon, Serge. Protoporphyrin IX fluorescence photobleaching is a useful tool to predict the response of rat ovarian cancer following hexaminolevulinate photodynamic therapy. *Lasers Surg Med* **2008**, *40*, 332-341.

24 McIlroy, Brian W., Mann, Thomas S., Dysart, Jonathan S. & Wilson, Brian C. The effects of oxygenation and photosensitizer substrate binding on the use of fluorescence photobleaching as a dose metric for photodynamic therapy. *Vibrational Spectroscopy* **2002**, *28*, 25-35.

25 Wilson, B. C., Patterson, M. S. & Lilge, L. Implicit and explicit dosimetry in photodynamic therapy: a New paradigm. *Lasers Med Sci* **1997**, *12*, 182-199.

26 Zhu, Timothy C. & Finlay, Jarod C. The role of photodynamic therapy (PDT) physics. *Med. Phys*. **2008**, *35*, 3127-3136.

27 Patterson, Michael S., Madsen, Steen J. & Wilson, Brian C. Experimental tests of the feasibility of singlet oxygen luminescence monitoring in vivo during photodynamic therapy. *Journal of Photochemistry and Photobiology*, B: Biology **1990**, *5*, 69-84.

28 Strekowski, Lucjan & Editor. Heterocyclic Polymethine Dyes; Synthesis, Properties and Applications. [In: Top. *Heterocycl. Chem*., **2008**; 14]. (*Springer GmbH*, **2008**).

29. James. Nadine S., Chen, Yihui, Joshi, Penny., Ohulchanskyy, Tymish Y., Ethirajan, M., Henary, M., Strekowsk, L. and Pandey Ravindra K. Evaluation of polymethine dyes as potential probes for near infrared fluorescence imaging of tumors: Part-I. *Theranostics*, **2013,** 3, 692-702.

30. James. Nadine S., Ohulchanskyy, Tymish Y Chen, Y., Joshi, Penny, Zheng, Xiang, Goswami, Lalit N. and Pandey Ravindra K. Comparative tumor imaging and PDT efficacy of HPPHconjugated in the mono- and di-forms to various polymethine cyanine dyes: Part-2. *Theranostics*, **2013**, 3, 703-718.

31 Henderson, Barbara W., Busch, Theresa M. & Snyder, John W. Fluence rate as a modulator of PDT mechanisms. *Lasers Surg Med* **2006**, *38*, 489-493.

32 Achilefu, Samuel, Bornhop, Darryl J., Raghavachari, Ramesh & Editors. Molecular Probes for Biomedical Applications II. (Proceedings held in San Jose, CA 21-22 January **2008**). [In: Proc. SPIE, **2008**; 6867]

33 Henderson, Barbara W., Gollnick, Sandra O., Snyder, John W., Busch, Theresa M., Kousis, Philaretos C., Cheney, Richard T. & Morgan, Janet. Choice of Oxygen-Conserving Treatment Regimen Determines the Inflammatory Response and Outcome of Photodynamic Therapy of Tumors. *Cancer Res*. **2004**, *64*, 2120-2126.

34 Pogue, Brian W., Sheng, Chao, Benevides, Juan, Forcione, David, Puricelli, Bill, Nishioka, Norm & Hasan, Tayyaba. Protoporphyrin IX fluorescence photobleaching increases with the use of fractionated irradiation in the esophagus. *Journal of biomedical optics* **2008**, *13*, 034009/034001-034009/034010.

35 Robinson, Dominic J., De, Bruijn Henriette S., Van, Der Veen Nynke, Stringer, Mark R., Brown, Stanley B. & Star, Willem M. Fluorescence photobleaching of ALA-induced protoporphyrin IX during photodynamic therapy of normal hairless mouse skin: the effect of light dose and irradiance and the resulting biological effect. *Photochem. Photobiol*. **1998**, *67*, 140-149.

36 Robinson, Dominic J., De, Bruijn Henriette S., Van, Der Veen Nynke, Stringer, Mark R., Brown, Stanley B. & Star, Willem M. Protoporphyrin IX fluorescence photobleaching during ALA-mediated photodynamic therapy of UVB-induced tumors in hairless mouse skin. *Photochem. Photobiol*. **1999**, *69*, 61-70.

37 Sheng, Chao, Hoopes, P. Jack, Hasan, Tayyaba & Pogue, Brian W. Photobleaching-based dosimetry predicts deposited dose in ALA-PpIX PDT of rodent esophagus. *Photochem. Photobiol*. **2007**, *83*, 738-748.

38 van, Veen Robert L. P., Aalders, Maurice C. G., Pasma, Kasper L., Siersema, Peter D., Haringsma, Jelle, van, de Vrie Wim, Gabeler, Edward E. E., Robinson, Dominic J. & Sterenborg, Henricus J. C. M. In situ light dosimetry during photodynamic therapy of Barrett's esophagus with 5-aminolevulinic acid. *Lasers Surg Med* **2002**, *31*, 299-304.

39 Patterson, Michael S. & Wilson, Brian C. A theoretical study of the influence of sensitizer photobleaching on depth of necrosis in photodynamic therapy. *Proc. SPIE-Int. Soc. Opt. Eng*. **1994**, *2133*, 208-219.

40 Patterson, Michael S., Wilson, Brian C. & Graff, Ronald. In vivo tests of the concept of photodynamic threshold dose in normal rat liver photosensitized by aluminum chlorosulfonated phthalocyanine. *Photochem. Photobiol*. **1990**, *51*, 343-349.

41 Wilson, Brian C., Weersink, Robert A. & Lilge, Lothar. 529-561 (Marcel Dekker, Inc.).