

## Assessing daylight & low-dose rate photodynamic therapy efficacy, using biomarkers of photophysical, biochemical and biological damage metrics in situ



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### ABSTRACT

**Background:** Sunlight can activate photodynamic therapy (PDT), and this is a proven strategy to reduce pain caused by conventional PDT treatment, but assessment of this and other alternative low dose rate light sources, and their efficacy, has not been studied in an objective, controlled pre-clinical setting. This study used three objective assays to assess the efficacy of different PDT treatment regimens, using PpIX fluorescence as a photophysical measure, STAT3 cross-linking as a photochemical measure, and keratinocyte damage as a photobiological measure.

**Methods:** Nude mouse skin was used along with in vivo measures of photosensitizer fluorescence, keratinocyte nucleus damage from pathology, and STAT3 cross-linking from Western blot analysis. Light sources compared included a low fluence rate red LED panel, compact fluorescent bulbs, halogen bulbs and direct sunlight, as compared to traditional PDT delivery with conventional and fractionated high fluence rate red LED light delivery.

**Results:** Of the three biomarkers, two had strong correlation to the PpIX-weighted light dose, which is calculated as the product of the treatment light dose ( $J/cm^2$ ) and the normalized PpIX absorption spectra. Comparison of STAT3 cross-linking to PpIX-weighted light dose had an  $R = 0.74$ , and comparison of keratinocyte nuclear damage  $R = 0.70$ . There was little correlation to PpIX fluorescence. These assays indicate most of the low fluence rate treatment modalities were as effective as conventional PDT, while fractionated PDT showed the most damage.

**Conclusions:** Daylight or artificial light PDT provides an alternative schedule for delivery of drug-light treatment, and this pre-clinical assay demonstrated that in vivo assays of damage could be used to objectively predict a clinical outcome in this altered delivery process.

### 1. Introduction

Conventional photodynamic therapy (PDT) using 5-aminolaevulinic acid (ALA) is commonly used to treat actinic keratosis (AK) [1,2], with some investigational and some approved uses in squamous cell carcinoma (SCC) [3], and basal cell carcinoma (BCC) [4–6]. Despite ALA-PDT being highly effective to treat AKs and non-melanoma skin cancers, patients often report moderate to severe pain associated to the

treatment [7–10], and this has been viewed as one of the more problematic issues in acceptance of the treatment. The source of pain in ALA-PDT is believed to be from protoporphyrin IX (PpIX) production or accumulation in nerve endings [11] which leads to damage during illumination. This pain has been related to the PpIX concentration in AK lesions [12].

To date, several studies have reported daylight-mediated PDT as effective as conventional PDT to treat AK lesion grade I with reduced

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pain [13]. Since “daylight PDT” consists in a low rate PDT light delivered by the sun for extended periods of time with either none or low incubation time of the photosensitizer, then the PpIX is produced at the same time that it is photobleached away in the treatment process [14–17]. So, the delivery process of daylight PDT appears effective with sunlight, however at the same time this process of no incubation time with continuous irradiation could also be easily achieved with lamps in a clinical setting, where the light delivery and patient behavior might be better controlled. The development of this paradigm with low pain but effective light delivery in a clinical setting could be a successful conduit for increased use of PDT, if demonstrated to be equally effective as traditional PDT delivery.

In the present study, we investigated the hypothesis that “daylight PDT” using different light sources could be as effective as conventional PDT, using the well-established model of normal nude mouse skin [18,19]. The study used three in vivo biomarkers of PDT treatment efficacy, including PpIX fluorescence assessed by fiberoptic dosimetry [15,20–22], damage to epidermal keratinocytes assessed by pathology [23], and induction of STAT3 cross-linking [24–26] as assessed by molecular analysis of biopsy samples, and examined these in response to different light sources as well as conventional PDT and fractionated PDT [27].

## 2. Materials and methods

### 2.1. PDT treatment

All animal studies were approved by Dartmouth College Institutional Animal Care and Use Committee (IACUC) and conducted in accordance with institutional PHS and OLAW guidelines.

Seventy female nude mice were used (Charles River Laboratories, Wilmington, MA) and separated into 10 animals/group with groups: (1) untreated control (no ALA, no light), (2) sunlight, (3) halogen, (4) Compact Fluorescent Light (CFL), (5) red LED, (6) traditional PDT, and (7) fractionated light PDT (fPDT). Groups 5–7 used the same red (633 nm) LED panel (Omnilux revive, Sydney, Australia), but group 5 was set at a lower fluence rate. All groups were prescribed a total light dose of  $\sim 78 \text{ J/cm}^2$ , except for the sun ( $107 \text{ J/cm}^2$ ), which could not be easily controlled. The “effective” light dose was calculated by using a PpIX-weighted irradiance [28,29] (details in supplementary data).

A volume of 20  $\mu\text{L}$  Levulan® Kerastick® (20% of ALA) solution was applied topically to the back of each mouse. In the “daylight” groups (sun, halogen, CFL, and LED), the animals received light immediately after ALA application for 2.5 h. In the single-illumination group (PDT), the animals were illuminated after 2.5 h of ALA application. In the fPDT group, the animals were illuminated twice, first after 1.25 h of dark incubation, and then again at 2.5 h after the initial ALA application (Fig. 1). The ALA was not reapplied during nor between any illuminations. During “daylight” treatment, the animals were awake for the duration of the procedure, while during PDT and fPDT, the mice were

anaesthetized with isoflurane.

Light treatments were given for the different light sources, directly measuring the optical irradiance and time of light delivered to the surface of the tissue. The spectrum of each source relative to the absorption spectrum of PpIX was used to calculate the PpIX-weighted irradiance, which was the product of the normalized PpIX absorption spectrum and the treatment light as multiplied wavelength by wavelength and then integrated together [28,29]. This process does not take into account individual variation in PpIX production, but rather just estimates an ‘effective’ irradiance related to the light source, which allows comparison between light sources from the theoretical efficiency of how they should excite PpIX. Details of the measurement systems and exact calculations are in Supplementary data. Time integrated irradiance was then reported as the delivered light dose.

### 2.2. Fluorescence dosimetry measurements

Active dosimetry of PpIX was done using optical measurements of remitted fluorescent intensity were acquired with both 405 nm laser (blue channel) and 635 nm laser (red channel) excitation. These measurements were corrected with white light reflectance measurements, to correct for attenuation due to individual variation. All optical measurements were collected using a previously reported point-probe dosimetry system and the analysis was carried out by applying an iterative Monte Carlo-based look-up-table (LUT) fitting algorithm [22]. For “daylight PDT” groups, the measurements were done (1) prior ALA administration (*Pre-ALA*) and (2) after light illumination (*Post-PDT*). For the regular PDT group (PDT), measurements were performed at three time points: (1) prior to ALA (*Pre-ALA*), (2) immediately before PDT (*Pre-PDT*) (data not showed), and (3) after treatment (*Post-PDT*). For the fractionated light illumination group (fPDT), the measurements were performed at five time points: (1) prior to ALA application (*Pre-ALA*), (2) before 1st light fraction (*Pre-1st PDT*), (3) after 1st light fraction (*Post-1st PDT*), (4) before 2nd light fraction (*Pre-2nd PDT*), and (5) after 2nd light fraction (*Post-2nd PDT*). All measurements were acquired with the probe gently in contact with the back of the mice, where the ALA was applied. The post-PDT PpIX fluorescence (*PpIX FL<sub>norm</sub>*) was normalized by subtracting the average skin auto-fluorescence obtained before ALA application ( $\overline{FL}_{\text{Pre-ALA}}$ ) from the average fluorescence obtained post-PDT (or post-2nd PDT, for fPDT group) ( $\overline{FL}_{\text{Post-PDT}}$ ) for each mouse and for both the blue and red excitation channels.

$$PpIX \text{ FL}_{\text{norm}} (a. u.) = \overline{FL}_{\text{Post-PDT}} - \overline{FL}_{\text{Pre-ALA}} \quad (1)$$

### 2.3. Western blot analysis

The proteins from skin were extracted immediately after light treatment using RIPA buffer containing protease and phosphatase inhibitors and 1 mM of PMSF. The skin was kept cooled by ice for approximately 20 min before electric homogenization, followed by centrifuge for 5 min at 13,000 rpm. The supernatant was transferred to a clean tube, and this sample volume was frozen at  $-20^\circ\text{C}$ . This was repeated for 5 mice/group.

Protein extracts (50  $\mu\text{g}$ ) were briefly heated at  $100^\circ\text{C}$  in  $\beta\text{ME}$ -containing buffer, separated on a polyacrylamide gel (4–15% Criterion™ TGX™, Bio-Rad), and transferred to 0.2  $\mu\text{m}$  PVDF membrane (Trans-Blot® Turbo™ Mini PVDF Transfer, Bio-Rad). On all gels, reference protein markers for molecular size detection (Precision Plus Protein Standards Kaleidoscope, Bio-Rad, #161-0375) were included. Non-specific interactions were blocked by incubating the membranes with 0.1% Tween 20, 5% powder milk in PBS for 1 h at room temperature. Membranes reacted overnight at  $4^\circ\text{C}$  with primary antibodies (anti-STAT3 C-20, Santa Cruz, 1:500; anti- $\beta$ -actin N-21, Santa Cruz, 1:500). Detection of the immune complexes were performed using a fluorescent secondary antibody, 1:15,000 (IRDye® 800CW Goat anti-Rabbit IgG, LI-

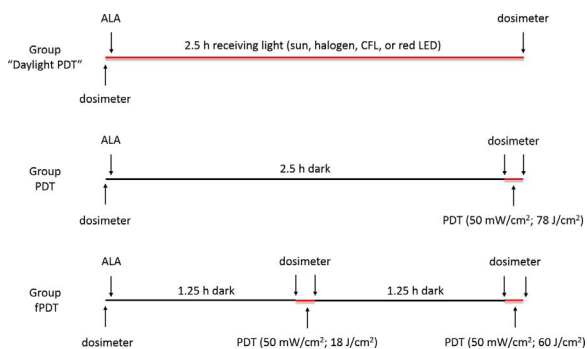


Fig. 1. Schematic of the different PDT regimens is shown with light delivery shown by the red lines, relative to the time of application of ALA to the skin (left side).

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