Photodynamic Therapy–Induced Immunosuppression in Humans Is Prevented by Reducing the Rate of Light Delivery

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Photodynamic therapy (PDT) of non-melanoma skin cancers currently carries failure rates of 10–40%. The optimal irradiation protocol is as yet unclear. Previous studies showed profound immunosuppression after PDT, which may compromise immune-mediated clearance of these antigenic tumors. Slower irradiation prevents immunosuppression in mice, and may be at least as effective as high-fluence-rate PDT in preliminary clinical trials. The photosensitizers 5-aminolaevulinic acid and/or methyl aminolaevulinate were applied to discrete areas on the backs of healthy Mantoux-positive volunteers, followed by narrowband red light irradiation (632 nm) at varied doses and fluence rates. Delayed type hypersensitivity (Mantoux) reactions were elicited at test sites and control sites to determine immunosuppression. Human ex vivo skin received low- and high-fluence-rate PDT and was stained for oxidative DNA photolesions. PDT caused significant, dose-responsive immunosuppression at high (75 mW cm⁻²) but not low (15 or 45 mW cm⁻²) fluence rates. DNA photolesions, which may be a trigger for immunosuppression, were observed after high-fluence-rate PDT but not when light was delivered more slowly. This study demonstrates that the current clinical PDT protocol (75 mW cm⁻²) is highly immunosuppressive. Simply reducing the rate of irradiation, while maintaining the same light dose, prevented immunosuppression and genetic damage and may have the potential to improve skin cancer outcomes.

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INTRODUCTION

Photodynamic therapy (PDT) for skin cancer comprises topical application of a photosensitizer, followed by irradiation with visible light in the presence of oxygen. PDT with systemic photosensitizers has been used to treat a range of internal malignancies, including lung, brain, esophagus, bladder, and head and neck cancers (Brown et al., 2004). Topical PDT, using the photosensitizing heme precursors 5-aminolaevulinic acid (ALA) or methylaminolaevulinate (MAL), is increasingly used to treat superficial basal cell carcinoma (BCC), Bowen’s disease (squamous cell carcinoma in situ), and premalignant actinic keratoses (Morton et al., 2002). Compared with surgery, ALA/MAL-PDT offers excellent functional and cosmetic outcomes and is a more practical option for the treatment of superficial but large diameter lesions, and for patients with large numbers of skin cancers.

Although ALA/MAL-PDT clearance rates of >90% have been found for smaller superficial BCCs (Haller et al., 2000), reported long-term clearance rates for BCC typically range from ~50–84% depending on lesion size and number of PDT sessions (Szeimies, 2007). Clearance rates of ~76% at 5 years have been reported after two PDT sessions for nodular BCCs (Rhodes et al., 2007) and ~80% at 12 months for Bowen’s disease (Morton et al., 2006), whereas actinic keratoses showed initial clearance of ~75% and recurrence of 20% at 12 months (Tschen et al., 2006). Hence, ALA/MAL-PDT using currently recommended protocols carries relatively high failure rates. Immune suppressed transplant recipients show even higher PDT failure rates, reflecting the key role of skin immunity in the resolution of these antigenic tumors (Dragieva et al., 2004).

For ALA/MAL-PDT of skin cancers, a maximum fluence rate of 200 mW cm⁻² is recommended (Babilas et al., 2006), however, there is no defined lower limit. At high-fluence rates, more rapid oxygen consumption likely outstrips the oxygen supply from surrounding blood vessels, whereas at low-fluence rates, tissue oxygenation remains at levels adequate for completion of the photodynamic process (Foster et al., 1991). Murine studies have shown that lower-fluence rates cause more efficient photobleaching and greater tissue
damage by topical ALA-PDT (Robinson et al., 1998), and yield better tumor kill after systemic PDT (Sitnik and Henderson, 1998; linuma et al., 1999). Preliminary human studies also suggest that ALA/MAL-PDT with lower-fluence rates may be as or more effective than current protocols (Langmack et al., 2001; Ericson et al., 2004; Cottrell et al., 2008). PDT is highly immunosuppressive in mice (Elmets and Bowen, 1986), with suggestion that this may be prevented or even reversed by lowering fluence rates (Sitnik and Henderson, 1998; Henderson et al., 2004). The immune effects of varying PDT fluence rates in humans are as yet unknown.

PDT is generally considered to have little carcinogenic risk (Morton et al., 2008). There are, however, reports of malignancies developing in ALA/MAL-PDT-treated fields (Wolf et al., 1997; Varma et al., 2000). The oxidative photoproduct 8-hydroxy-2′-deoxyguanosine (8oxoG) is a marker of DNA damage that is observed after UVA and UVB irradiation (Javeri et al., 2008). PDT induces 8oxoG in mice (Takahashi et al., 2005) and DNA strand breaks in human fibroblasts (Haylett et al., 2003), however, there are no reported studies in human skin.

The Mantoux reaction, a T-cell-mediated delayed type hypersensitivity response to intradermally injected tuberculin purified protein derivative (PPD), offers a practical and ethical model of in vivo skin immunity (Damian and Halliday, 2002). Using this model, we recently reported that topical ALA/MAL-PDT, delivered at 78 mW cm⁻², is highly immunosuppressive in humans (Matthews and Damian, 2010). We now report that ALA/MAL-PDT at high but not low-fluence rates is immunosuppressive and causes generation of 8oxoG lesions in humans.

RESULTS
High-fluence-rate MAL-PDT and ALA-PDT are immune suppressive
Fifteen volunteers of Fitzpatrick’s skin types II and III (Fitzpatrick, 1998; 8 men, 7 women, mean age 40 years, range 23–65) had mirrored templates on each side of their lower backs, which were treated with MAL, ALA, vehicle, or no lotion. One side of the back was irradiated with high-fluence-rate red light (fluence rate 75 mW cm⁻², total dose 37 J cm⁻²), whereas the other side remained light protected. MAL-PDT significantly suppressed Mantoux in duration (mean ± SEM ∆diameter 3.1 ± 0.7 mm, P = 0.003, n = 15; Figure 1a) corresponding to a ∆33% mean reduction in diameter. MAL-PDT also suppressed erythema (∆erythema index (EI)) 34.7 ± 9.8 erythema units, P = 0.03, n = 15; Figure 1b) equating to a 37% mean reduction in EI. Likewise, ALA-PDT significantly suppressed Mantoux diameter (∆diameter 3.6 ± 0.5 mm, P < 0.001, Figure 1a) corresponding to a 40% mean reduction in diameter. ALA-PDT EI was suppressed (∆EI 53.6 ± 9.6 erythema units, P < 0.001, Figure 1b) by 67% compared with control.

Neither photosensitizer caused immune suppression in the absence of irradiation. Mantoux reactions at unirradiated, MAL-treated sites did not differ from control Mantoux reactions (∆diameter 0.4 ± 0.1 mm, P = 0.08, Figure 1a; ∆EI 5.4 ± 5.3 erythema units, P = 1.0, Figure 1b). Similarly, ALA alone was not immunosuppressive (∆diameter 0.4 ± 0.1 mm, P = 0.07, Figure 1a; ∆EI 8.0 ± 5.4 erythema units, P = 1.0, Figure 1b). Sites receiving red light only did not show suppression of Mantoux diameter or erythema compared with unirradiated control sites (Figure 1). This was further investigated in three men and seven women (skin types II and III, mean age 40, range 26–74). At separate sites on the back, these volunteers received red light only (75 mW cm⁻²) with total doses of 37, 111, and 222 J cm⁻² (up to six times the standard therapeutic light dose) and MAL-PDT as a positive control (37 J cm⁻², 75 mW cm⁻²). Two additional sites served as unirradiated controls. Red light did not suppress Mantoux induration nor erythema at any dose (∆diameter −0.4 ± 0.2 mm, P = 0.4; 0.5 ± 0.2 mm, P = 0.3; −0.6 ± 0.5 mm, P = 1.0; and ∆EI 1.7 ± 6.8, P = 1.0; −8.8 ± 7.6, P = 1.0; −0.7 ± 7.8 P = 1.0 erythema units for 37, 111, and 222 J cm⁻², respectively). Again, MAL-PDT caused immunosuppression of ~20% in diameter (∆diameter 20% in diameter (∆diameter

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**Figure 1.** Photodynamic therapy (PDT; fluence rate 75 mW cm⁻² and total dose 37 J cm⁻²) with either methyl aminolaevulinate (MAL) or 5-aminolaevulinic acid (ALA) is highly immune suppressive in humans. Red light alone or with vehicle, or photosensitizer alone (no light) did not cause immunosuppression. Mantoux diameter and erythema at each treatment site was subtracted from that at the unirradiated control site to determine immunosuppression (∆diameter, ∆EI; Student’s paired t test, n = 15). EI, erythema index; VEH, vehicle.
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Figure 2. High-fluence-rate PDT (75 mW cm\(^{-2}\)) with methyl aminolaevulinate (MAL) caused significant, dose responsive suppression of Mantoux reactions. In contrast, low-fluence-rate MAL-PDT (15 mW cm\(^{-2}\)) was not immune suppressive at any dose (a, Δdiameter; b, ΔEI). Student’s paired t-test, \(n=15\). EI, erythemia index.

MAL-PDT is immune suppressive at high but not low fluence rates

Fifteen volunteers (skin types II and III, 7 men, 8 women, mean age 39, range 26–74) had MAL-PDT administered to each side of the lower back. On one side total doses of 10, 20, and 37 J cm\(^{-2}\) were delivered at a high-fluence rate of 75 mW cm\(^{-2}\). These three doses were also delivered to the contralateral back at a low-fluence rate of 15 mW cm\(^{-2}\). Mantoux reactions at each site were compared with unirradiated controls (no light, no MAL).

High-fluence-rate MAL-PDT significantly suppressed Mantoux reactions at total light doses of 10, 20, and 37 J cm\(^{-2}\) (Δdiameter 2.9 ± 0.6 mm, \(P=0.002\); ΔEI 34.5 ± 7.5 erythema units, \(P=0.002\)) and 20 J cm\(^{-2}\) (Δdiameter 1.9 ± 0.5 mm, \(P=0.006\); ΔEI 35.0 ± 6.4 erythema units, \(P=<0.001\)) but not 10 J cm\(^{-2}\) (Δdiameter 1.4 ± 0.5 mm, \(P=0.24\); ΔEI 23.3 ± 9.7 erythema units, \(P=0.15\); Figure 2). MAL-PDT delivered at a low-fluence rate (15 mW cm\(^{-2}\)) did not, however, suppress Mantoux reactions at any dose (37 J cm\(^{-2}\) Δdiameter −0.2 ± 0.4 mm, \(P=1.0\); ΔEI 13.7 ± 8.5 erythema units, \(P=0.7\); 20 J cm\(^{-2}\) Δdiameter −0.2 ± 0.4 mm, \(P=1.0\), ΔEI 1.5 ± 7.1 erythema units, \(P=1.0\); 10 J cm\(^{-2}\) Δdiameter 0.1 ± 0.4 mm, \(P=1.0\), ΔEI 3.4 ± 6.6 erythema units, \(P=1.0\); Figure 2).

Delivery of 37 J cm\(^{-2}\) at a fluence rate of 75 mW cm\(^{-2}\) takes ~8 minutes, hence, delivery of this dose at 15 mW cm\(^{-2}\) takes ~40 minutes, which is less feasible in clinical practice. Fifteen additional volunteers (skin types II and III, seven men, eight women, mean age 40, range 24-65) were thus recruited to compare the immune effects of an intermediate fluence rate (45 mW cm\(^{-2}\); irradiance time 13 minutes) to irradiance at 75 mW cm\(^{-2}\). High-fluence (75 mW cm\(^{-2}\)) MAL-PDT was immunosuppressive in this group of volunteers at a dose of 37 J cm\(^{-2}\) (Δdiameter 1.8 ± 0.5 mm, \(P=0.02\); ΔEI 24.7 ± 7.0 erythema units, \(P=0.02\)), but not at lower doses (20 J cm\(^{-2}\) Δdiameter 1.1 ± 0.4 mm, \(P=0.07\), ΔEI 30.5 ± 10.3 erythema units, \(P=0.05\); 10 J cm\(^{-2}\) Δdiameter 0.4 ± 0.3 mm, \(P=0.1\), ΔEI 16.9 ± 6.5 erythema units, \(P=0.11\), Figure 3).

MAL-PDT delivered with a fluence rate of 45 mW cm\(^{-2}\) did not suppress Mantoux reactions at any total light dose (37 J cm\(^{-2}\) Δdiameter −0.6 ± 0.5 mm, \(P=1.0\), ΔEI 13.0 ± 10.1 erythema units, \(P=1.0\); 20 J cm\(^{-2}\) Δdiameter −0.2 ± 0.4 mm, \(P=1.0\); Figure 3).
Figure 4. Human skin samples (duplicates from three volunteers) were treated with methylaminolaevulinate (MAL) only, or low-fluence (15 mW cm$^{-2}$) or high-fluence (75 mW cm$^{-2}$) MAL-photodynamic therapy. Staining for oxidative DNA photolesions (8-hydroxy-2'-deoxyguanosine) was performed at various time points up to 2 hours to assess DNA repair as well as damage. The percentage of epidermal area positively staining for 8-oxo at each treatment time point was compared with untreated control skin (unpaired two-tailed Student’s t-test, Bonferroni correction).

Figure 5. Human skin (bar = 100 μm) was stained for 8-hydroxy-2'-deoxyguanosine. Control (untreated) skin showed low-level baseline staining consistent with oxidative damage resulting from normal cellular metabolism (a). Methylaminolaevulinate (MAL)-photodynamic therapy (PDT)-treated skin using a high-fluence rate of 75 mW cm$^{-2}$, total light dose 37 J cm$^{-2}$, t= 60 minutes following irradiation showed significantly higher levels of oxidative DNA damage (b) compared with MAL-PDT-treated skin using a low-fluence rate (c, 15 mW cm$^{-2}$).

DISCUSSION

Consistent with our previous findings (Matthews and Damian, 2010), ALA-PDT was profoundly immunosuppressive in humans, measured by both Mantoux erythema and induration, using a high-fluence-rate protocol. ALA-PDT caused a higher level of immunosuppression (~50%) than MAL-PDT (~35%). ALA and MAL produce the same intracellular photosensitizer (PpIX), penetrate human skin to similar depths and produce a similar spatial distribution of PpIX in murine skin (de Bruijn et al., 2008). However, ALA produces relatively greater amounts of PpIX (Juzeniene et al., 2006) and higher levels of fluorescence with a slower time course than MAL in human skin (Lesar et al., 2009). This, together with greater local edema reported after ALA compared with MAL-PDT in mice (de Bruijn et al., 2008) and the possibility of different microscopic PpIX distributions following ALA and MAL application to human skin, could explain the greater immunosuppression observed after ALA-PDT in our study. We examined normal skin, but intralesional variations in PpIX distribution within tumors could also influence the immune effect of PDT (de Haas et al., 2008).

ALA/MAL-PDT using our protocol impaired local immunity to existing antigens (recall immunity), and could thus impair local antitumor immunity and tumor eradication. The effects of PDT on the development of immunity to new tumor antigens (primary immunity) are unknown. In our studies, immunosuppression was evident 6 days after PDT; however, it is unknown how long this effect persists in humans. In clinical practice, skin cancers receive ALA/MAL-PDT on two occasions 1 week apart (Morton et al., 2008), suggesting...
immune suppression for at least 2 weeks, but possibly longer given that PDT immunosuppression persists for at least 28 days in mice (Gollnick et al., 2001).

We found significant, dose responsive immune suppression with MAL-PDT delivered at 75 mW cm\(^{-2}\), which was completely prevented by simply lowering the fluence rate to deliver an identical total dose five times more slowly. An intermediate fluence rate of 45 mW cm\(^{-2}\), a more practical option requiring irradiation of only an additional 5 minutes beyond current clinical recommendations, also did not cause immunosuppression.

The putative central mechanism of PDT is generation of reactive oxygen species (ROS) by irradiation of a photosensitizer. PDT-induced ROS can then react with a range of cellular targets, including cell membrane lipids and mitochondria as well as DNA. Irradiation with lower doses can alter cell signalling and cytokine and receptor expression in the absence of cell death, suggesting that there are different dose responses for these ROS effects (Calzavarina-Pinton et al., 2007). In human fibroblasts treated with ALA-PDT, 70-95% of cell kill occurred without detectable DNA damage, suggesting that mitochondrial damage, which we did not directly examine, is also a key mechanism of cell death (Haylett et al., 2003).

In normal human skin, we found less oxidative DNA damage after low-fluence-rate MAL-PDT, although the effect of reduced fluence rate on DNA damage in tumor cells is unknown. ALA-based photosensitizers are more readily taken up by tumors than by normal skin, with a 10-fold increase in PpIX production in dysplastic or malignant lesions (Fritsch et al., 2004). It is thus possible that low-fluence-rate PDT might maintain efficacy of tumor kill by more selectively damaging tumor DNA, causing other ROS effects such as cytokine triggering and mitochondrial damage (Hilf, 2007) and inducing tumor cell apoptosis (Singh et al., 2010). We examined only total DNA and it remains possible that our low-fluence rate may not have spared oxidative damage to mitochondria.

Despite the possibility that reduced oxidative DNA damage, as we observed here, might impair efficacy, studies using low-fluence-rate protocols have so far demonstrated comparable tumor clearance rates when compared with high-fluence-rate regimens in actinic keratoses treated with ALA and 580-650 nm or 580-690 nm light sources (Ericson et al., 2004) and in superficial BCCs treated with ALA and narrowband 633 nm light (Cottrell et al., 2008). This implies that high-fluence rates might not be required for efficacy. In BCCs, photodynamic efficiency (loss of light absorbance as photosensitizer is used in the photodynamic process) decreased with increased fluence rate; 20 mW cm\(^{-2}\) of narrowband red light was most efficient (Cottrell et al., 2008). A 635 nm red light source was as effective in the treatment of superficial BCCs at 12.6 J cm\(^{-2}\), delivered at 7 mW cm\(^{-2}\) over 30 minutes, as standard treatment (37 J cm\(^{-2}\) over 8 minutes; i.e., 75 mW cm\(^{-2}\); Langmack et al., 2001). There is a suggestion that low-irradiance ambulatory ALA-PDT (12 mW cm\(^{-2}\)) may also be effective (Moseley et al., 2006). Similarly, high clearance rates for actinic keratoses were reported with lower fluence rates (30 mW cm\(^{-2}\); Ericson et al., 2004) or even irradiation using low-intensity Copenhagen sunlight (Wiegel et al., 2008). Higher numbers of neutrophils, greater inflammation, and greater expansion of tumor-specific T cells are seen after low-irradiance, low-dose systemic PDT compared with a high-irradiance, high-dose regimen in mice (Kousis et al., 2007). Fractionation of irradiation has also been suggested to improve the efficacy of topical ALA-PDT for superficial BCC (de Haas et al., 2006), with suggestion that neutrophils induced by systemic PDT may be key mediators of anti-tumor immunity (Kousis et al., 2007) and anti-tumor efficacy (de Bruijn et al., 2006) in this setting. The role and fluence rate-dependence of neutrophil infiltration in topical ALA/MAL-PDT in humans is unknown.

Ultraviolet radiation induces 8oxoG photolesions that are products of oxidative damage, and are highly mutagenic if left unrepaired (Kozmin et al., 2005). We found that high but not low-fluence-rate MAL-PDT induced these photolesions. UV-induced cyclobutane pyrimidine dimers are a key trigger of UV-induced immunosuppression (Kuchel et al., 2005) and it has been suggested that 8oxoG may have similar effects. Inhibition of ROS-mediated damage, such as 8oxoG, prevents immunosuppression (Yao et al., 2009). More recently it has been shown that UV-induced platelet activating factor and cis-urocanic acid both induce 8oxoG formation and cause immunosuppression, whereas inhibition of these mediators reduced 8oxoG and immunosuppression (Sreevidya et al., 2010). It is not known yet whether enhanced repair of 8oxoG helps to prevent immunosuppression, but topical application of the 8oxoG repair enzyme 8 oxoguanosine DNA glycosylase reduced tumor size and progression in photoinduced tumor models (Wulff et al., 2008). Hence, ROS-mediated damage, such as 8oxoG, is a key molecular mechanism causing immunosuppression (Halliday, 2010).

In our study, the immunosuppression following high-fluence-rate ALA/MAL-PDT may reflect oxidative DNA damage, which at lower fluence rates is no greater than the expected baseline level of oxidative damage that results from normal cellular metabolism (Javeri et al., 2008). Additional studies are now indicated to better evaluate the cellular and cytochemical changes involved in PDT-induced immunomodulation, to improve PDT efficacy in skin cancer, but possibly also to use immune suppressive effects in the treatment of immune-mediated disorders such as psoriasis (Morton et al., 2008).

Topical PDT at high but not low-fluence rates causes both immunosuppression and induction of oxidative DNA photolesions in human skin. As both immunosuppression and DNA damage are recognized tumor promoters, this suggests that simply irradiating more slowly may have the potential to reduce tumor recurrence and boost cure rates. Further studies are now urgently needed to assess the role of PDT-immunosuppression in PDT treatment failures and the mechanisms of cell toxicity following low-fluence-rate PDT. Using randomized controlled trials, we then need to determine the comparative efficacy of low irradiance rate PDT, so that we can establish and recommend to clinicians the optimal PDT protocol.
MATERIALS AND METHODS

Mantoux testing
We recruited healthy Mantoux positive hospital staff and students, who had previously been vaccinated with Bacille Calmette-Guerin. Volunteers were not taking immunosuppressive or photosensitizing medications and were asked to avoid sun exposure for 4 weeks before and throughout the study, as even small UV exposures can suppress skin immunity (Damian and Halliday, 2002). Approval for these studies was provided by the Sydney South West Area Health Service and University of Sydney Ethics Committees and all participants provided written informed consent. All experiments were performed in accordance with the precepts established by the Helsinki Declaration.

Volunteers were initially Mantoux tested with three graded doses of tuberculin PPD (Tubersol; Sanofi-Pasteur, Toronto, ON, Canada) to determine both Mantoux positivity and the appropriate PPD dose to elicit a Mantoux diameter of ~8 mm. PPD was diluted with normal saline to a volume of 0.1 ml and injected intradermally on the lateral lower back with 31-gauge needles. Mantoux reactions were assessed 72 hours later using the ‘pen method’, which measures Mantoux diameter of induration in two perpendicular planes (Bouros et al., 1991). The EI at each Mantoux site and at adjacent skin was measured in triplicate using a portable reflectance meter (Dia-stron, Andover, UK). The EI of adjacent skin was then subtracted from the EI overlying Mantoux reactions to determine Mantoux-induced erythema, and to adjust for any PDT-induced erythema (Damian and Halliday, 2002).

Photodynamic therapy
Templates were created on each side of the lower back using Confeel Plus Ulcer Dressing (Coloplast A/S, 3050 Humlebaek, Denmark) to create discrete 3 × 2 cm areas separated by 1 cm. Treatment allocation at each square was randomized based on order of study entry, with a mirror image of treatment site allocation on the contralateral back. In all, 40 mg cm⁻² of the photosensitizers 20% ALA in white soft paraffin (Biosynth AG, Staad, Switzerland) and MAL 16% cream (Mettix: Galderma, Belrose, New South Wales, Australia) or vehicle (emulsifying ointment) were applied to the various sites, which were then occluded for 3 hours under Tegaderm dressings (3M Healthcare, St Paul, MN) and light protected with aluminum foil. The creams were then removed and test sites exposed to varied fluence rates and total light doses from an Aktilite CL128 LED array (PhotoCure, ASA, Oslo, Norway). The light source emitted red light with a peak of 632 nm as measured at 1 nm intervals with an Optronics OL754 spectroradiometer (Optronics, Orlando, FL) calibrated against the spectro-radiometer (International Light Technologies, Peabody, MA), calibrated against the spectro-radiometer. After irradiation, all treatment sites remained light protected with aluminum foil for a further 24 hours.

Measurement of immnosuppression and data analysis
At 72 hours after irradiation each of the treatment and control sites were injected with PPD, and the resulting Mantoux reactions quantitated by diameter and erythema measurements 72 hours thereafter. Immunosuppression was calculated as the difference between control site (no photosensitizer, no light) and treatment site Mantoux reactions. Results are shown as mean ± SEM; significance was determined by Bonferroni corrected paired Student’s t-tests with P<0.05 considered significant.

Measurement of PDT-induced DNA photolesions
Three healthy adults undergoing abdominoplasty (n=2) or breast reduction (n=1) consented for their excised skin to be used in the study. Skin was collected in normal saline, trimmed of excess fat, washed in chlorhexitine, rinsed with phosphate-buffered saline (Invitrogen, Calsbad, CA), and cut into 3 cm squares, which received MAL only (no light), low-fluence-rate MAL-PDT (15 mW cm⁻²), high-fluence-rate MAL-PDT (75 mW cm⁻²), or no treatment (control). MAL (16%, 40 mg cm⁻²) was applied and occluded with Tegaderm. The skin was then placed into separate petri dishes containing RPMI-1640 medium (Sigma-Aldrich, St Louis, MO) supplemented with 10% fetal bovine serum and 100 U ml⁻¹ penicillin, 0.1 mg ml⁻¹ streptomycin, and 250 ng ml⁻¹ amphotericin B (Sigma-Aldrich). Petri dishes were light protected with aluminum foil and incubated for 3 hours at 37 °C in 5% CO₂ in air. MAL was removed using phosphate-buffered saline and specimens were rinsed twice in phosphate-buffered saline and cut into 5 mm squares. The two PDT groups were then exposed to 37 J cm⁻² of red light (Aktilite) with a fluence rate of 15 or 75 mW cm⁻². Duplicate skin pieces were collected immediately or after various times of incubation in the media described above, snap frozen in OCT (Tissue-Tek, Sakura, Zoeterwoude, the Netherlands) and stored at −70 °C. Frozen sections (6-μm thick) were stained by immunohistochemistry using mouse monoclonal anti-8oxoG antibody (Trevigen, Gaithersburg, MD), as we have described previously (Javeri et al., 2008). Separate pieces of skin were treated with MAL-PDT (37 J cm⁻², 75 mW cm⁻²) or solar-simulated UV (4 J cm⁻²), and stained for cyclobutane pyrimidine dimers (Kamiya Biomedical, Seattle, WA).

The area of positively stained epidermis was determined by image analysis (Image, Wayne Rasband, National Institutes of Health). The average percentage staining for each treatment group at the five time points was then calculated and compared with the average of the control by Student’s unpaired t-tests (Boneferroni correction; P<0.05 considered significant).

CONFLICTS OF INTEREST
The authors state no conflict of interest.

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