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Blue light rescues mice from potentially fatal *Pseudomonas aeruginosa* burn infection: efficacy, safety, and mechanism of action

Short title: Blue light for lethal burn infections in mice.

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Abstract

Blue light has attracted increasing attention due to its intrinsic antimicrobial effect without the addition of exogenous photosensitizers. However, the use of blue light for wound infections has not been established yet. In this study, we demonstrated the efficacy of blue light at 415 nm for treatment of acute, potentially lethal *Pseudomonas aeruginosa* burn infections in mice. *In vitro* studies demonstrated that the inactivation rate of *P. aeruginosa* cells by blue light was approximately 35-fold faster than that of keratinocytes (P=0.0014). Transmission electron microscopy revealed blue light mediated intracellular damage to *P. aeruginosa* cells. Fluorescence spectroscopy suggested that coproporphyrin III or/and uroporphyrin III are possibly the intracellular photosensitive chromophores associated with the blue light inactivation of *P. aeruginosa*. *In vivo* studies using an in vivo bioluminescence imaging technique and the area-under-the-bioluminescence-time-curve (AUBC) analysis showed that a single exposure of blue light at 55.8 J/cm^2, applied 30 min after bacterial inoculation to the infected mouse burns, reduced the AUBC by an approximately 100-fold in comparison with untreated and infected mouse burns (P<0.0001). Histological analysis and TUNEL assay indicated no significant damages in the mouse skin exposed to blue light at the effective antimicrobial dose. Survival analysis revealed that blue light increased the survival rate of infected mice from 18.2% to 100% (P<0.0001). In conclusion, blue light therapy may offer an effective and safe alternative to conventional antimicrobial therapy for *P. aeruginosa* burn infections.

Introduction

Burns are one of the most common and devastating forms of trauma (4, 34, 38, 46). Data from the National Center for Injury Prevention and Control in the United States show that approximately 2 million fires are reported each year which result in 1.2 million people with burn injuries. Significant thermal injuries induce a state of immunosuppression that predisposes burn patients to infectious complications (4). Burn wound infections are one of the most important and potentially serious complications that occur in the acute period following injury (33, 34, 38). Over the last several decades, Gram-negative organisms have emerged as the most common etiologic agents of invasive infection by virtue of their large repertoire of virulence factors and antimicrobial resistance traits (1, 4). Among Gram-negative organisms, *Pseudomonas aeruginosa* is one of the more feared bacterial pathogens because it is often resistant to current therapeutic modalities (33). Emerging antimicrobial resistance trends in burn wound bacterial pathogens represent a serious therapeutic challenge for clinicians caring for burn patients (4). As a result, a major research effort has been led to find alternative antimicrobial approaches to which, it is hypothesized, bacteria will not be easily able to develop resistance. In a recent paper published in *Nature Reviews Microbiology*, Dr. Karen Bush and a group of 30 scientists from academia and industry pointed out that, "*The investigation of novel non-antibiotic approaches for the prevention of and protection against infectious diseases should be encouraged, and such approaches must be high-priority research and development projects.*"(3)

As a non-antibiotic approach, the development of light-based antimicrobial therapy, including photodynamic therapy (PDT) (5, 8, 10, 12, 21, 25) and ultraviolet C (UVC) irradiation therapy (6, 9, 11, 13, 42, 43), have both been extensively investigated as alternatives to conventional antibiotics. Advantages of light-based antimicrobial therapies include equal killing effectiveness regardless of antibiotic resistance. However, one major disadvantage of PDT as a two-part (photosensitizer + light) combination approach is the challenge of introducing photosensitizers into certain bacteria (44) and into infected tissues and less than perfect selectivity of many photosensitizers for microbial cells over host tissue. The use of UVC irradiation, on the other hand, has different limitations due to its detrimental effects on mammalian cells and possible damage to host tissue including carcinogenesis (13).

Another novel light-based approach, termed blue light therapy, is attracting increasing attention due to its intrinsic antimicrobial effect without the addition of exogenous photosensitizers (7, 16, 17, 29, 31). In addition, it is accepted that blue light is much less detrimental to mammalian cells than ultraviolet irradiation (26, 27). Blue light has already been used clinically for treatment of inflammatory acne (15, 24, 32, 47). However, the use of blue light for wound infections has not been established. The majority of the publications on the antimicrobial effect of blue light have been confined to *in vitro* studies (16, 17, 20, 28, 29). There have been (rather surprisingly) no published preclinical or clinical reports to demonstrate blue light therapy for wound infections.

92 In this study, we investigated the use of blue light for mouse burns infected with *Pseudomonas*
93 *aeruginosa*. To best of our knowledge, this study is the first *in vivo* study on the use of blue light
94 for wound infections.

Materials and methods

Light source

The light source we used was a Omnilux clear-U™ light emitting diode (LED) array (Photo Therapeutics, Inc., Carlsbad, CA) that emitted blue light at a center wavelength of 415 nm with a full width at half maximum (FWHM) of 20 nm (Fig. 1). The irradiance of blue light on the target surface was adjusted by manipulating the distance between the LED array aperture and the target (cell culture or mouse burns), and was measured using a PM100D power/energy meter (Thorlabs, Inc., Newton, NJ).

Pseudomonas aeruginosa strain and culture conditions

The *P. aeruginosa* strain that we used was ATCC 19660 (strain 180), which causes septicemia after intraperitoneal injection (37) and which has been shown to be invasive in mice with skin burns (30). The stable bioluminescent variant of the strain (strain Xen 05) carried the entire bacterial lux operon integrated in their chromosomes for stable luciferase expression, which allowed it to be used for bioluminescent imaging (strain Xen 05 was a kind donation from Xenogen Inc, Alameda, CA) (36).

The bacteria were grown in brain heart infusion (BHI) medium supplemented with 50 µg/mL kanamycin in an orbital incubator (37°C; 100 rpm) to an optical density of 0.6 to 0.8 at 600 nm, which corresponds to approximately 10^8 cells/mL. The suspension was centrifuged,

washed with phosphate-buffered saline (PBS), and resuspended in PBS at the same density for experimental use.

Keratinocytes and culture conditions

The human keratinocyte cell line (HaCaT) (2) were cultured in 75-cm^3 tissue culture flasks in 20 mL Dulbecco's modified Eagle's medium supplemented with 10% heat-inactivated fetal bovine serum, penicillin (100 units/mL) and streptomycin (100 µg/mL) (Sigma, St. Louis, MO). Cells were incubated at 37 °C, 95% air, 5% CO_2 in a humidified incubator for 2–3 days until the cell monolayer became confluent. Growth medium was replaced every 3 days. Upon reaching at least 70% confluence, the cells were washed with PBS and trypsinized for 10 min at 37 °C with 0.25% trypsin, 0.02% ethylenediamine tetraacetic acid (Sigma). The cell suspension was centrifuged, washed with PBS, and resuspended in HEPES buffer (catalog # A14291 DJ, Life Technologies Corp., Grand Island, NY) to a defined cell density (measured by hemocytometer) for experimental use.

Blue light inactivation of *P. aeruginosa in vitro*

Three (3) mL *P. aeruginosa* suspension at 10^8 CFU/mL in PBS was placed into 35-mm petri dishes. The suspension was irradiated with blue light LED array at an irradiance of 19.5 mW/cm^2 with the lid of the petri dish removed. During the irradiation, the *P. aeruginosa* suspension was stirred by a mini-magnetic bar (Fisher Scientific Co., Norcross, GA). Aliquots of 40 µL of the

suspension were withdrawn at 0, 12, 24, 48, 72, and 96 min, respectively, when 0, 14.0, 28.0, 56.1, 84.2, and 109.9 J/cm^2 blue light had been delivered. Colony forming units (CFU) were then determined by serial dilution on BHI agar plates by the method of Jett et al (23). Colonies were allowed to grow for 18-24 h at 37 °C. Experiments were performed in triplicate.

Blue light irradiation of keratinocytes in vitro

Three (3) mL keratinocyte suspension at 10^6 cell/mL in HEPES buffer was placed into 35-mm petri dishes at room temperature (21 °C). The suspension was irradiated with the blue light LED array at an irradiance of 19.5 mW/cm^2 with the lid of the petri dish removed. During the irradiation, the keratinocytes suspension was stirred by a mini-magnetic bar. Aliquots of 40 µL of the suspension were withdrawn at 0, 12, 24, 48, 72, and 96 min, respectively, when 0, 14.0, 28.0, 56.1, 84.2, and 109.9 J/cm^2 blue light had been delivered. Viable counts were determined immediately by mixing each sample with an equal volume of 0.4% (w/v) trypan blue and the mixture transferred to a haemocytometer. The cell survival percentage was calculated as the ratio of the number of viable cells (unstained cells) to the total number of cells. The experiments were performed in triplicate.

Transmission electron microscopy

Untreated and blue light treated *P. aeruginosa* cells were fixed in 2.5% glutaraldehyde + 2% paraformaldehyde immediately after blue light illumination and stored overnight at 4 °C. After

spinning down (1200 rpm, 10 min) and decanting the fixative, 0.1 M sodium cacodylate buffer (pH 7.2) was added to the pellets. After fixation, hot agar (2% in distilled water, heated to boiling) was immediately added to each pellet. Once the agar had hardly solidized, the cell pellets were then processed routinely, as any other tissue, for transmission electron microscope (TEM). The cell pellets were postfixed in 2% OsO_4 in sodium cacodylate buffer, dehydrated in a graded alcohol series, and embedded in Epon t812 (Tousimis, Rockville, MD). Ultrathin sections were cut on a Reichert-Jung Ultracut E microtome (Vienna, Austria), collected on uncoated 200 mesh copper grids, stained with uranyl acetate and lead citrate, and examined on a Philips CM-10 transmission electron microscope (Eindhoven, The Netherlands).

Fluorescence spectroscopy

To identify the porphyrins within *P. aeruginosa* cells, an overnight *P. aeruginosa* culture was centrifuged, washed with PBS, centrifuged again, and the supernatant removed. The *P. aeruginosa* pellets were added to 1 mL of a mixture of 0.1 M NaOH/1% sodium dodecyl sulfate (SDS) and allowed to stand in the dark for 1 day. Fluorescence of the dissolved *P. aeruginosa* pellets in NaOH/SDS (in a cuvette 1 cm thick) was measured on a fluorimeter (Fluoromax 3, SPEX Industries, Edison, NJ), with excitation at 405 nm and emission scanned from 580 to 700 nm.

***P. aeruginosa* burn infection in mice**

Adult female BALB/c mice, 7–8 week old and weighing 17–21 g, were obtained from Charles River Laboratories (Wilmington, MA). The animals were housed one per cage with access to food and water ad libitum, and were maintained on a 12-hour light/dark cycle under a room temperature of around 21 °C and a relative humidity range of 30-70%. All animal procedures were approved by the Subcommittee on Research Animal Care (IACUC) of the Massachusetts General Hospital and were in accordance with the guidelines of the National Institutes of Health (NIH).

Before the creation of burns, the mice were anesthetized by intraperitoneal (I.P.) injection of a ketamine-xylazine cocktail, shaved on the dorsal surfaces. Burns were incurred by applying a pre-heated (≈95°C) brass block to the dorsal surface of each mouse for 3 sec, resulting in nonlethal, full-thickness, third-degree burns measuring approximately 1.2-cm ×1.2-cm. Five min after burn incurrence, 60-µL bacterial suspension containing 3×10^6 CFU was topically applied on to the eschar of each burn.

Bioluminescence imaging

The setup consists of an ICCD camera (Model C2400-30H, Hamamatsu Photonics, Bridgewater, NJ), a camera controller, an imaging box, an image processor (C5510-50, Hamamatsu), and a color monitor (PVM 1454Q, Hamamatsu). Light-emitting diodes are mounted inside the imaging box and supply the light required for obtaining dimensional imaging of the sample. Under photo counting mode, a clear image can be obtained even under extremely low-light levels by detecting and integrating individual photons one by one.

Prior to imaging, mice were anesthetized by I.P. injections of ketamine/xylazine cocktail. Mice were then placed on an adjustable stage in the specimen chamber, and the infected burns were positioned directly under the camera. A gray-scale background image of each wound was made, and this was followed by a photon count of the same region. This entire burn photon count was quantified as relative luminescence units (RLUs) and was displayed in a false color scale ranging from pink (most intense) to blue (least intense).

Blue light therapy of mouse burns infected with *P. aeruginosa*

Blue light was initiated at 30 min after bacterial inoculation with the irradiance of 14.6 mW/cm^2. Mice were given a total light exposure of up to 55.8 J/cm^2 in aliquots with bioluminescence imaging taking place after each aliquot of light. To record the time course of the extent of bacterial infection, bacterial luminescence from mouse burns was measured daily after blue light therapy until the infections were cured (characterized by the disappearance of bacterial luminescence) or the burns healed.

TUNEL Assay

Mouse skin was exposed to blue light at the antimicrobial dose of 55.8 J/cm^2. Skin biopsies were taken before and at 0, 24, and 48 h after blue light exposure. The biopsies were preserved in 10% phosphate-buffered formalin (Fisher Sci., USA) for 18-24 h, processed, and then embedded in paraffin. Serial tissue sections of 4 µm thickness were subjected to TUNEL assay

using the FragEL DNA Fragmentation Detection Kit (EMD Millipore, MA, USA), according to the manufacturer's instructions. Briefly, following deparaffinization and rehydration, sections were permeabilized with proteinase K for 20 min and then incubate with reaction mixture containing terminal deoxynucleotidyl transferase (TdT) and fluorescein labeled and unlabeled deoxynucleotides for 2 h at room temperature, followed by washing with TBS, and coverslipped with mounting media including diamidino-2-phenylindole (DAPI) (SlowFade Gold anti-fade reagent, Invitrogen CA). Negative controls were treated by substituting dH2O for the TdT enzyme in the reaction mixture. Stained samples were observed by confocal microscopy (Fluoview FV1000-MPE, Olympus Corporation, Tokyo, Japan) by using FITC as the fluor and DAPI as nuclear counterstain and images were acquired using Olympus Fluoview FV10-ASW software, (Version 3.0a, Olympus Corporation, Tokyo, Japan).

Since penetration depth is less than 1 mm at blue light which is confined to epidermis (2), we only examined the epidermal cell DNA fragmentation by TUNEL staining.

Statistical analyses

The cell inactivation rates (slopes of the survival curves) were compared for statistical significance using a Student *t*-test. In a two-dimensional coordinate system, the area-under-the-bioluminescence curve (AUBC) data, which represent the time courses of bacterial luminescence of the mouse burns and also a common approach for the analysis of antimicrobial effect of drugs (18), were calculated using numerical integration (14). Difference in the AUBC

between the untreated control and the blue light treated groups was compared for statistical significance also using a Student *t* test. Kaplan-Meier survival curves were compared by the use of a log-rank test. *P* values of <0.05 were considered significant for all statistical analyses.

Results

Blue light selectively inactivated *P. aeruginosa in vitro* over keratinocytes.

The results for the blue light inactivation of *P. aeruginosa* and keratinocytes *in vitro* are shown in Fig. 2. The inactivation curves approximately followed the first-order kinetics (48), a linear relation between the log-transformed cell survival fraction $\log_{10}(N/N_0)$ and blue light exposure H, i.e.,

$$\log_{10}\frac{N}{N_0} = -k_H H \quad (1)$$

where, N is the CFU count at the blue light exposure H, N_0 is the initial CFU count, k_H is the cell inactivation rate coefficient (or the slope of the inactivation curve) (39).

When 109.9 J/cm^2 blue light had been delivered (96 min illumination at the irradiance of 19.5 mW/cm^2), approximately 7.64-$\log_{10}$-cycle CFU inactivation of *P. aeruginosa* was achieved. In contrast, the inactivation rate for HaCaT was much slower than that for *P. aeruginosa* under the same blue light irradiation condition. When 109.9 J/cm^2 blue light had been delivered, only 0.16-$\log_{10}$-cycle loss of viability of HaCaT cells was observed (Fig. 1), resulting in a 7.48-$\log_{10}$ inactivation selectivity of *P. aeruginosa* cells over HaCaT cells. The mean inactivation rate coefficients (k_H) of *P. aeruginosa* and HaCaT were 0.067 and 0.002 cm^2/J, respectively, indicating an approximately 34-fold faster inactivation rate of *P. aeruginosa* by blue light than HaCaT cells (P= 0.0014).

Transmission electron microscopy (Fig. 3) revealed apparent steps in blue light mediated inactivation of *P. aeruginosa*, beginning with the development of vacuoles within the cytoplasm (Fig. 3B), release of cytoplasmic material to the surrounding environment (Fig. 3C), and, finally, significant cytoplasmic disruption (Fig. 3D).

Intracellular coproporphyrin III or/and uroporphyrin III were associated with the blue light inactivation of *P. aeruginosa*.

The fluorescence spectrum (excitation at 405 nm) of the *P. aeruginosa* cells dissolved in NaOH/SDS is shown in Fig. 4. The spectrum peaked at 613 and 667 nm, which is very close to the typical fluorescence emissions of coproporphyrin III and uroporphyrin III at the same excitation of 405 nm (40), suggesting that coproporphyrin III or/and uroporphyrin III within the *P. aeruginosa* cells were the photosensitizing chromophores associated with the antimicrobial effect of blue light.

Blue light rescued mice from otherwise lethal *P. aeruginosa* infection.

Fig. 5A and B show the successive bioluminescence images of representative full thickness mouse burns (1.2-cm×1.2-cm) infected with 3×10^6 CFU of luminescent *P. aeruginosa*, with and without blue light therapy, respectively. Blue light (415 nm) was delivered at 30 min after bacterial inoculation. Bacterial luminescence was completely eliminated after 55.8 J/cm^2 blue light had been delivered (62 min illumination at the irradiance of 14.6 mW/cm^2) (Fig. 5A), while

in the untreated mouse burn, infection steadily developed with the time (Fig. 5B) and the mouse died at 72 h (day 3) after bacterial inoculation. Luminescent *P. aeruginosa* was detected in the blood culture of the dead mouse, indicating that the mouse died of sepsis.

Fig. 5C shows the average reduction in bacterial luminescence from 11 mice each of which was exposed to blue light. The *in vivo* inactivation curve also approximately followed the first-order kinetics. After 55.8 J/cm^2 blue light had been delivered, an average of 3.5-log$_{10}$-cycle of reduction of bacterial luminescence was achieved in a light dose dependent manner, with the bacterial inactivation rate coefficient k_H approximately 0.064 cm^2/J.

Fig. 5D shows the time courses of the mean bacterial luminescence (RLU) from day 1 to day 3 of the blue light-treated mice (n=11) and untreated ones (n=11). The RLU values of the blue light treated group are significantly lower than those of the untreated group from day 1 to day 3 (P=0.0008 on day 1; P=6.11×10^{-5} on day 2; P=0.049 on day 3).The AUBC of bioluminescence time course (from day 1 to day 2, i.e., the time period before most of the mortalities occurred) were (1.19±2.57) ×10^5 and (8.91±4.53) ×10^6 for blue light-treated mice and untreated mice, respectively (P<0.0001, Fig. 5E), indicating an approximately 100-fold reduction of AUBC resulted from blue light acute treatment (exposure?).

297 Overall, all mice (*n*=11) survived after blue light acute treatment while only 18.2% (2 out of 11)
298 mice survived without blue light acute treatment(*P*<0.0001, Fig. 5F). Most of the mortalities (6
299 out of 9) occurred on day 3 (72 h) after bacterial inoculation.

300

301 **No significant and irreversible damages were observed in the mouse skin exposed to blue**
302 **light at the effective antimicrobial dose.**

303

304 Fig. 6A shows hematoxylin and eosin stained histological sections of a representative mouse
305 skin exposed to blue light at the dose of 55.8 J/cm^2. Immediately after the blue light exposure,
306 swelling of nucleus of basal cells and slight edema in the upper of dermis were observed.
307 However, at 48 h after blue light, the epithelium returned to its normal composition and the
308 collagenous fibers were lined up in order in dermis.

309

310 Fig. 6B shows the representative results of TUNEL assay of mouse skin exposed to blue light. A
311 blue light exposure of 55.8 J/cm^2 led to almost no apoptotic cells in the epidermis immediately
312 after blue light exposure (only one positive TUNEL was observed in the confocal image).
313 Similarly, lack of positive- TUNEL epidermal cells after 24 or 48 h were observed (only one
314 positive TUNEL was observed in each confocal image). These results demonstrated that blue
315 light irradiation at the therapeutic antimicrobial dose is safe and no adverse effect in terms of
316 DNA damage observed up to 48 h of post-blue light treatment.

317

Discussion

We report here for the first time an *in vivo* study demonstrating the efficacy of blue light for *P. aeruginosa* burn infection in mice. The study may serve as an initial effort in the pursuit of utilizing a novel therapeutic option, blue light therapy, for wound infections, especially those caused by multi-drug-resistant bacteria. Thus, the first and most important impact is through opening a new area of study on a different therapeutic regimen for wound infections.

It was found in the *in vitro* study that *P. aeruginosa* was much more susceptible to blue light inactivation than were HaCaT cells. As a result, there exists a therapeutic window where *P. aeruginosa* can be selectively inactivated by blue light while the host tissue cells can be preserved.

We found from the *in vivo* study that blue light at 415 nm wavelength, when applied at 30 min after bacterial inoculation, effectively reduced the *P. aeruginosa* burden in mouse burns and prevented otherwise lethal bacteremia in mice. As the *P. aeruginosa* strain we used was lethal to mice. If without treatment, the bacteria can invade deep into the mouse tissue and, subsequently, the blood stream within hours, and cause bacteremia and mortality of the animals. Therefore, we initiated blue light therapy very soon (at 30 min) after bacterial inoculation. Histological analysis and TUNEL assay revealed no significant and reversible damages in the mouse skin exposed to blue light at the effective antimicrobial dose.

338

339 An interesting finding of the present study is the equal susceptibilities of *P. aeruginosa* cells to
340 blue light inactivation *in vitro* and *in vivo*. The bacterial inactivation rate coefficients were 0.067
341 and 0.064 cm^2/J for *in vitro* and *in vivo*, respectively. In our previously studies on the
342 antimicrobial PDT for wound infections (5, 12, 35), we observed that, to achieve an equivalent
343 amount of inactivation of microorganisms, orders of magnitude higher light exposures (also
344 higher doses of photosensitizers) were required for *in vivo* than *in vitro*. One possible major
345 factor responsible for this phenomenon is that the host tissue and cells compete with the
346 microorganisms for binding the exogenous photosensitizer, resulting in a reduced efficacy of *in*
347 *vivo* microorganism inactivation where the microorganisms are embedded in tissue. On the
348 contrary, it is suggested that blue light inactivation of bacteria is through the photo excitation
349 of naturally occurring porphyrins within the bacterial cells, which act as endogenous
350 photosensitizers. Therefore, the competition from surrounding host tissue and cells for binding
351 photosensitizers does not exist. One more advantage of antimicrobial blue light therapy is the
352 highly selective inactivation of bacterial cells over mammalian cells, because blue light targets
353 the photosensitizing porphyrins only within the bacterial cells, while mammalian cells (in the
354 absence of porphyria or added 5-aminolevulanic acid) do not contain free porphyrins.

355

356 We also investigated the mechanism of blue light inactivation of *P. aeruginosa*. TEM images
357 showed that blue light mediated damages to *P. aeruginosa* cells started from the development
358 of vacuoles within the cytoplasm, implying that the damage was associated with the

359 intracellular chromophores excited by blue light. By using fluorescence spectroscopy, we
360 observed that the emission maxima of the *P. aeruginosa* pellets dissolved in NaOH/SDS were
361 613 nm and 667 nm at the excitation of 405 nm. This emission spectrum is very close to those
362 of uroporphyrin III (emission maxima 618 nm and 670 nm) and coproporphyrin III (emission
363 maxima 615 nm and 674 nm) (40). Therefore, it is likely that the photosensitizing porphyrins
364 within *P. aeruginosa* cells are uroporphyrin III or coproporphyrin III, or both uroporphyrin III
365 and coproporphyrin III exist within *P. aeruginosa* cells. To further identify and quantify the
366 intracellular porphyrins, future studies are warranted by using techniques such as high-
367 performance liquid chromatography (HPLC) or capillary electrophoresis together with authentic
368 porphyrin standards (22)

369

370 As we stated earlier, we understand that there remain many questions unanswered. One more
371 question that will have to be addressed in the future study is "Can bacterial cells develop
372 resistance to blue light inactivation?" To our knowledge this question has not yet been
373 experimentally addressed. The possible development of bacterial resistance to PDT has been
374 studied. After repeated cycles of partial inactivation followed by regrowth, different bacterial
375 species failed to develop resistance to PDT after 10 (41) or even 20 cycles (19). It is commonly
376 accepted that PDT acts at multiple sites within bacterial cells (structural proteins, enzymes,
377 nucleic acids, unsaturated lipids etc) (45), and, therefore, would offer less potential for the
378 development of bacterial resistance than conventional antibiotics, which are usually single-
379 target specific. As the mechanism of antimicrobial effect of blue light is suggested to be similar

to that of PDT, one can expect that the potential of bacterial resistance development to blue light is also less than that of conventional antibiotics. However, at the very least it will be necessary to repeatedly deliver sub-eradication doses of blue light to susceptible cultures with regrowth between cycles to investigate whether resistant clones can be selected, or mutants with lower accumulation of porphyrins or increased blue light damage repairing enzymes can be produced.

In addition, more studies are also warranted to deepen the understanding of the approach of blue light therapy, for example, the comparison of the susceptibilities to blue light inactivation in vitro between pathogenic bacteria and host cells, including not only keratinocytes, but also fibroblast, endothelial cell, macrophage, and muscle cells; evaluation of the effects of blue light on the phagocytosis and ROS production of macrophage in vitro; determination of whether the wound pathogenic bacteria can develop resistance to blue light by repeated cycles of sub-total killing and re-growth *in vitro*; identification of the maximum safe exposure of mice to blue light in this model.

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Author Contributions

Conceived and designed the experiments: TD AG YH CKM MSV GPT, MRH. Performed the experiments: TD AG YH MS. Analyzed the data: TD AG YH MRH. Wrote the paper: TD AG MRH.

References

1. **Azzopardi, E. A., S. M. Azzopardi, D. E. Boyce, and W. A. Dickson.** 2011. Emerging gram-negative infections in burn wounds. J Burn Care Res **32:**570-576.
2. **Boukamp, P., R. T. Petrussevska, D. Breitkreutz, J. Hornung, A. Markham, and N. E. Fusenig.** 1988. Normal keratinization in a spontaneously immortalized aneuploid human keratinocyte cell line. J Cell Biol **106:**761-771.
3. **Bush, K., P. Courvalin, G. Dantas, J. Davies, B. Eisenstein, P. Huovinen, G. A. Jacoby, R. Kishony, B. N. Kreiswirth, E. Kutter, S. A. Lerner, S. Levy, K. Lewis, O. Lomovskaya, J. H. Miller, S. Mobashery, L. J. Piddock, S. Projan, C. M. Thomas, A. Tomasz, P. M. Tulkens, T. R. Walsh, J. D. Watson, J. Witkowski, W. Witte, G. Wright, P. Yeh, and H. I. Zgurskaya.** 2011. Tackling antibiotic resistance. Nat Rev Microbiol **9:**894-896.
4. **Church, D., S. Elsayed, O. Reid, B. Winston, and R. Lindsay.** 2006. Burn wound infections. Clin Microbiol Rev **19:**403-434.
5. **Dai, T., V. J. Bil de Arce, G. P. Tegos, and M. R. Hamblin.** 2011. Blue dye and red light, a dynamic combination for prophylaxis and treatment of cutaneous Candida albicans infections in mice. Antimicrob Agents Chemother **55:**5710-5717.
6. **Dai, T., B. Garcia, C. K. Murray, M. S. Vrahas, and M. R. Hamblin.** 2012. UVC Light Prophylaxis for Cutaneous Wound Infections in Mice. Antimicrob Agents Chemother **56:**3841-3848.
7. **Dai, T., A. Gupta, C. K. Murray, M. S. Vrahas, G. P. Tegos, and M. R. Hamblin.** 2012. Blue light for infectious diseases: Propionibacterium acnes, Helicobacter pylori, and beyond? Drug Resist Updat.
8. **Dai, T., Y. Y. Huang, and M. R. Hamblin.** 2009. Photodynamic therapy for localized infections--state of the art. Photodiagnosis Photodyn Ther **6:**170-188.
9. **Dai, T., G. B. Kharkwal, J. Zhao, T. G. St Denis, Q. Wu, Y. Xia, L. Huang, S. K. Sharma, C. d'Enfert, and M. R. Hamblin.** 2011. Ultraviolet-C light for treatment of Candida albicans burn infection in mice. Photochem Photobiol **87:**342-349.
10. **Dai, T., G. P. Tegos, Z. Lu, L. Huang, T. Zhiyentayev, M. J. Franklin, D. G. Baer, and M. R. Hamblin.** 2009. Photodynamic therapy for Acinetobacter baumannii burn infections in mice. Antimicrob Agents Chemother **53:**3929-3934.
11. **Dai, T., G. P. Tegos, T. G. St Denis, D. Anderson, E. Sinofsky, and M. R. Hamblin.** 2011. Ultraviolet-C irradiation for prevention of central venous catheter-related infections: an in vitro study. Photochem Photobiol **87:**250-255.
12. **Dai, T., G. P. Tegos, T. Zhiyentayev, E. Mylonakis, and M. R. Hamblin.** 2010. Photodynamic therapy for methicillin-resistant Staphylococcus aureus infection in a mouse skin abrasion model. Lasers Surg Med **42:**38-44.
13. **Dai, T., M. S. Vrahas, C. K. Murray, and M. R. Hamblin.** 2012. Ultraviolet C irradiation: an alternative antimicrobial approach to localized infections? Expert Rev Anti Infect Ther **10:**185-195.
14. **Davis, P. J., and P. Rabinowitz.** 2007. Methods of numerical integration, 2nd ed. Dover Publications, Mineola, N.Y.
15. **Elman, M., M. Slatkine, and Y. Harth.** 2003. The effective treatment of acne vulgaris by a high-intensity, narrow band 405-420 nm light source. J Cosmet Laser Ther **5:**111-117.
16. **Enwemeka, C. S., D. Williams, S. K. Enwemeka, S. Hollosi, and D. Yens.** 2009. Blue 470-nm light kills methicillin-resistant Staphylococcus aureus (MRSA) in vitro. Photomed Laser Surg **27:**221-226.

17. **Enwemeka, C. S., D. Williams, S. Hollosi, D. Yens, and S. K. Enwemeka.** 2008. Visible 405 nm SLD light photo-destroys methicillin-resistant Staphylococcus aureus (MRSA) in vitro. Lasers Surg Med **40:**734-737.
18. **Firsov, A. A., and H. Mattie.** 1997. Relationships between antimicrobial effect and area under the concentration-time curve as a basis for comparison of modes of antibiotic administration: meropenem bolus injections versus continuous infusions. Antimicrob Agents Chemother **41:**352-356.
19. **Giuliani, F., M. Martinelli, A. Cocchi, D. Arbia, L. Fantetti, and G. Roncucci.** 2010. In vitro resistance selection studies of RLP068/Cl, a new Zn(II) phthalocyanine suitable for antimicrobial photodynamic therapy. Antimicrob Agents Chemother **54:**637-642.
20. **Guffey, J. S., and J. Wilborn.** 2006. In vitro bactericidal effects of 405-nm and 470-nm blue light. Photomed Laser Surg **24:**684-688.
21. **Hamblin, M. R., and T. Hasan.** 2004. Photodynamic therapy: a new antimicrobial approach to infectious disease? Photochem Photobiol Sci **3:**436-450.
22. **Hamblin, M. R., J. Viveiros, C. Yang, A. Ahmadi, R. A. Ganz, and M. J. Tolkoff.** 2005. Helicobacter pylori accumulates photoactive porphyrins and is killed by visible light. Antimicrob Agents Chemother **49:**2822-2827.
23. **Jett, B. D., K. L. Hatter, M. M. Huycke, and M. S. Gilmore.** 1997. Simplified agar plate method for quantifying viable bacteria. Biotechniques **23:**648-650.
24. **Kawada, A., Y. Aragane, H. Kameyama, Y. Sangen, and T. Tezuka.** 2002. Acne phototherapy with a high-intensity, enhanced, narrow-band, blue light source: an open study and in vitro investigation. J Dermatol Sci **30:**129-135.
25. **Kharkwal, G. B., S. K. Sharma, Y. Y. Huang, T. Dai, and M. R. Hamblin.** 2011. Photodynamic therapy for infections: clinical applications. Lasers Surg Med **43:**755-767.
26. **Kleinpenning, M. M., T. Smits, M. H. Frunt, P. E. van Erp, P. C. van de Kerkhof, and R. M. Gerritsen.** 2010. Clinical and histological effects of blue light on normal skin. Photodermatol Photoimmunol Photomed **26:**16-21.
27. **Liebmann, J., M. Born, and V. Kolb-Bachofen.** 2010. Blue-light irradiation regulates proliferation and differentiation in human skin cells. J Invest Dermatol **130:**259-269.
28. **Lipovsky, A., Y. Nitzan, A. Gedanken, and R. Lubart.** 2010. Visible light-induced killing of bacteria as a function of wavelength: implication for wound healing. Lasers Surg Med **42:**467-472.
29. **Maclean, M., S. J. MacGregor, J. G. Anderson, and G. Woolsey.** 2009. Inactivation of bacterial pathogens following exposure to light from a 405-nanometer light-emitting diode array. Appl Environ Microbiol **75:**1932-1937.
30. **Markley, K., and E. Smallman.** 1968. Protection by vaccination against Pseudomonas infection after thermal injury. J Bacteriol **96:**867-874.
31. **McDonald, R., S. J. Macgregor, J. G. Anderson, M. Maclean, and M. H. Grant.** 2011. Effect of 405-nm high-intensity narrow-spectrum light on fibroblast-populated collagen lattices: an in vitro model of wound healing. J Biomed Opt **16:**048003.
32. **Morton, C. A., R. D. Scholefield, C. Whitehurst, and J. Birch.** 2005. An open study to determine the efficacy of blue light in the treatment of mild to moderate acne. J Dermatolog Treat **16:**219-223.
33. **Polavarapu, N., M. P. Ogilvie, and Z. J. Panthaki.** 2008. Microbiology of burn wound infections. J Craniofac Surg **19:**899-902.
34. **Rafla, K., and E. E. Tredget.** 2011. Infection control in the burn unit. Burns **37:**5-15.

35. **Ragas, X., T. Dai, G. P. Tegos, M. Agut, S. Nonell, and M. R. Hamblin.** 2010. Photodynamic inactivation of Acinetobacter baumannii using phenothiazinium dyes: in vitro and in vivo studies. Lasers Surg Med **42:**384-390.
36. **Rocchetta, H. L., C. J. Boylan, J. W. Foley, P. W. Iversen, D. L. LeTourneau, C. L. McMillian, P. R. Contag, D. E. Jenkins, and T. R. Parr, Jr.** 2001. Validation of a noninvasive, real-time imaging technology using bioluminescent Escherichia coli in the neutropenic mouse thigh model of infection. Antimicrob Agents Chemother **45:**129-137.
37. **Rosenthal, S. M.** 1967. Local and systemic therapy of pseudomonas septicemia in burned mice. Ann Surg **165:**97-103.
38. **Rowley-Conwy, G.** 2010. Infection prevention and treatment in patients with major burn injuries. Nurs Stand **25:**51-52, 54, 56-58 passim.
39. **Sinton, L. W., C. H. Hall, P. A. Lynch, and R. J. Davies-Colley.** 2002. Sunlight inactivation of fecal indicator bacteria and bacteriophages from waste stabilization pond effluent in fresh and saline waters. Appl Environ Microbiol **68:**1122-1131.
40. **Strauss, W. S., R. Sailer, H. Schneckenburger, N. Akgun, V. Gottfried, L. Chetwer, and S. Kimel.** 1997. Photodynamic efficacy of naturally occurring porphyrins in endothelial cells in vitro and microvasculature in vivo. J Photochem Photobiol B **39:**176-184.
41. **Tavares, A., Carvalho CM, Faustino MA, Neves MG, Tomé JP, Tomé AC, Cavaleiro JA, Cunha A, Gomes NC, Alves E, Almeida A.** 2010. Antimicrobial photodynamic therapy: study of bacterial recovery viability and potential development of resistance after treatment. Mar Drugs. **8:**91-105.
42. **Taylor, G. J., J. P. Leeming, and G. C. Bannister.** 1993. Effect of antiseptics, ultraviolet light and lavage on airborne bacteria in a model wound. J Bone Joint Surg Br **75:**724-730.
43. **Thai, T. P., D. H. Keast, K. E. Campbell, M. G. Woodbury, and P. E. Houghton.** 2005. Effect of ultraviolet light C on bacterial colonization in chronic wounds. Ostomy Wound Manage **51:**32-45.
44. **Wainwright, M.** 1998. Photodynamic antimicrobial chemotherapy (PACT). The Journal of antimicrobial chemotherapy **42:**13-28.
45. **Wainwright, M.** 2010. 'Safe' photoantimicrobials for skin and soft-tissue infections. Int J Antimicrob Agents **36:**14-18.
46. **Weber, J., and A. McManus.** 2004. Infection control in burn patients. Burns **30:**A16-24.
47. **Wheeland, R. G., and S. Dhawan.** 2011. Evaluation of self-treatment of mild-to-moderate facial acne with a blue light treatment system. J Drugs Dermatol **10:**596-602.
48. **Xiong, R., G. Xie, A. E. Edmondson, and M. A. Sheard.** 1999. A mathematical model for bacterial inactivation. Int J Food Microbiol **46:**45-55.

536 **Figure legends**

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538 **Fig. 1** Emission spectrum of Omnilux™ blue LED.

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541 **Fig. 2** Dose response of blue light inactivation of *P. aeruginosa* and keratinocytes *in vitro*.

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Fig. 3 Transmission electron microscopy of *P. aeruginosa* cells. (A) Untreated *P. aeruginosa* cells; Bar= 100 nm. (B)-(D) *P. aeruginosa* cells after being exposed to 109.9 J/cm^2 blue light: (B) Development of vacuoles within the cytoplasm; Bar=100 nm. (C) Release of cytoplasmic material to the surrounding environment; Bar=500 nm. (D) Complete disappearance of cytoplasm; Bar=100 nm.

Fig. 4 Fluorescence spectrum of *P. aeruginosa* cell pellets from overnight culture dissolved in NaOH/SDS. Excitation wavelength 405 nm.

Fig. 5 (A)-(B) Successive bacterial luminescence images of representative mouse burns infected with 3×10^6 CFU of luminescent *P. aeruginosa*, with and without blue light (415 nm) prophylaxis, respectively. Blue light was delivered at 30 min after bacterial inoculation. (C) Dose response of mean bacterial luminescence of mouse burns infected with 3×10^6 CFU of *P. aeruginosa* and exposed to blue light (415 nm) at 30 min after bacterial inoculation (n=11). Bars: standard deviation. (D) Time courses of mean bacterial luminescence of the infected skin abrasions with (n=11) and without blue light exposure (n=11 at day 1 and day 2, n= 4 at day 3), respectively. Bars: standard deviation. RLU values of blue light group vs. RLU values of untreated groups: day 1, P=0.0008; day 2, P=6.11×10^{-5}; day 3, P=0.049. (E) Mean areas under the bioluminescence versus time curves (from day 1 to day 2 in the two-dimensional coordinate system in panel D), representing the overall bacterial burden of mouse wounds. Bars: standard deviation. (F) Kaplan-Meier survival curves of blue light treated mouse burns (n=11) and untreated mouse burns (n=11) (P<0.0001).

564 **Fig. 6** (A) Hematoxylin and eosin stained histological sections of skin samples from a
565 representative mouse exposed to blue light at a dose of 55.8 J/cm^2. Skin samples were taken
566 before blue light, 0 h, 24 h, and 48 h after blue light exposure, respectively. BAR: 200 µm. (B)
567 TUNEL analyses of DNA damage in the same mouse skin showing in panel (A) (100X). Skin
568 samples were taken before blue light, 0 h, 24 h, and 48 h after blue light exposure, respectively.
569 Immunofluorescence of fluorescein and DAPI are represented by green and blue pseudo-color
570 respectively. DAPI is used for nuclear counter stain. Arrows: positive -TUNEL cells.

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573 Figure 1

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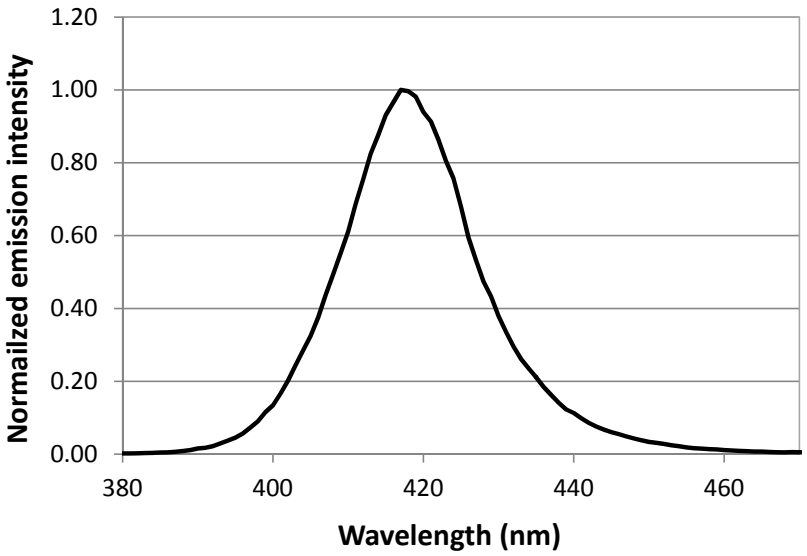

576 Figure 2

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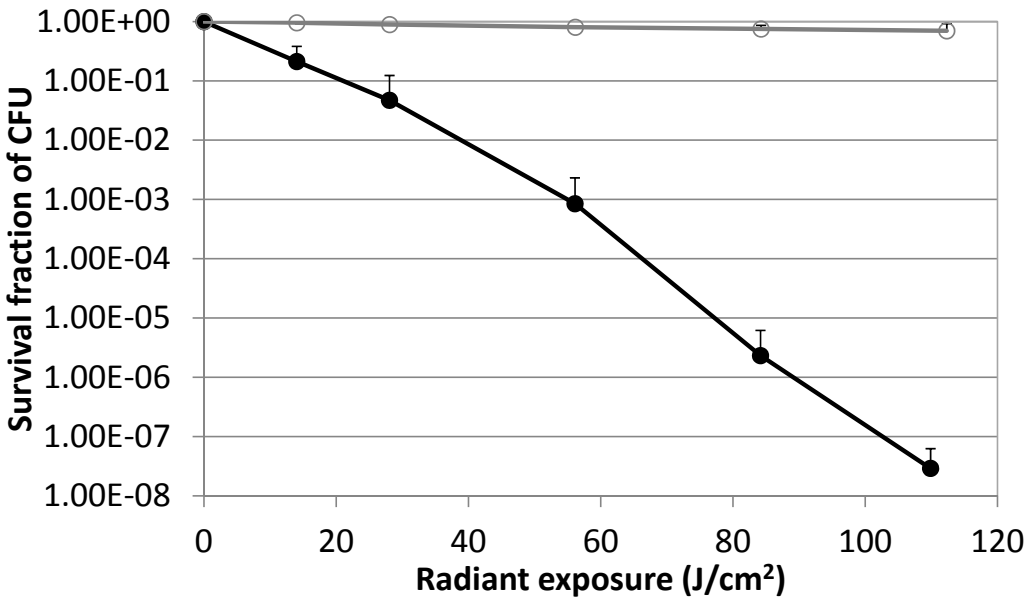

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584 Figure 3

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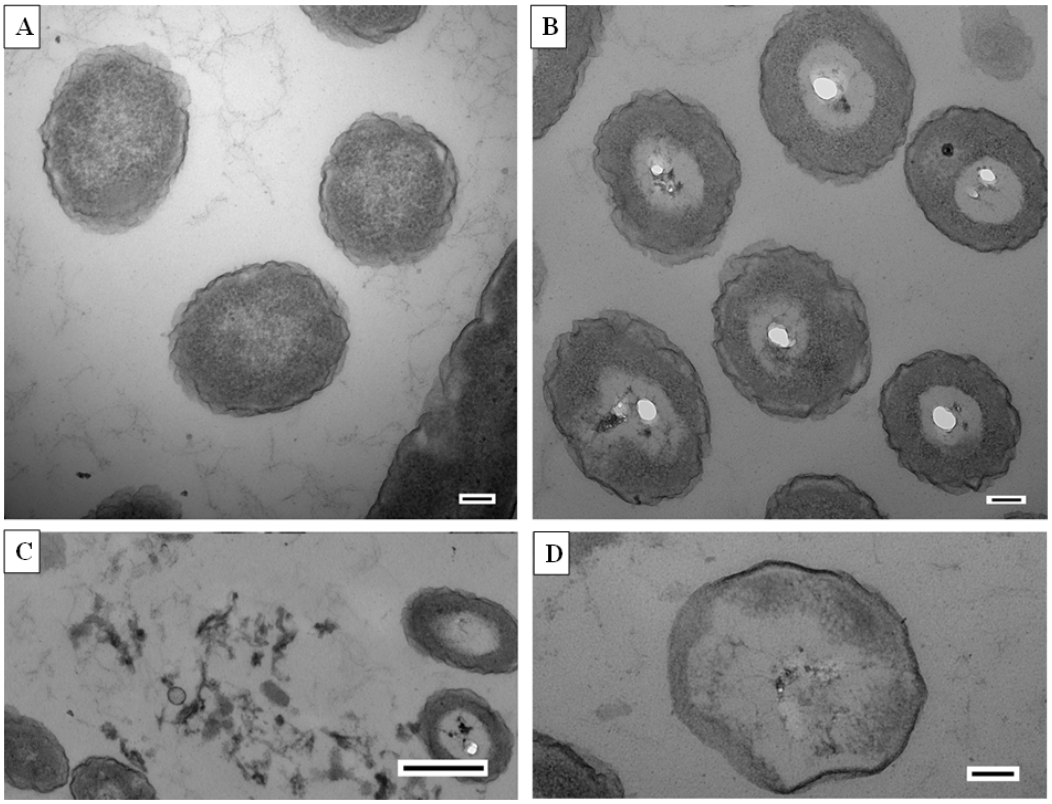
586 

587 Figure 4

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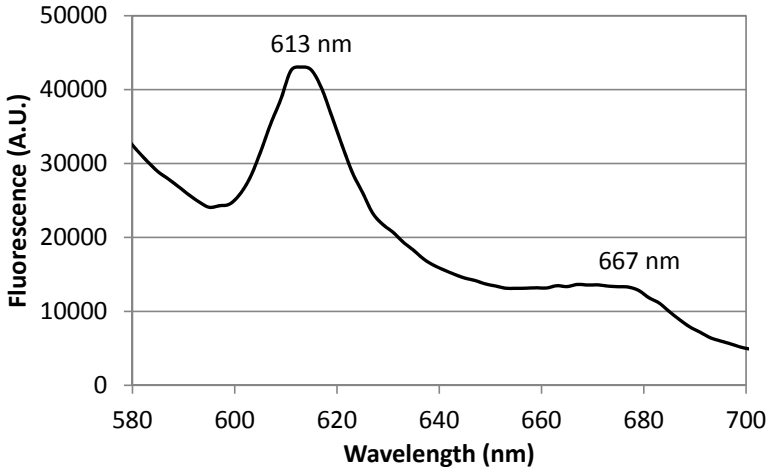

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592 Figure 5

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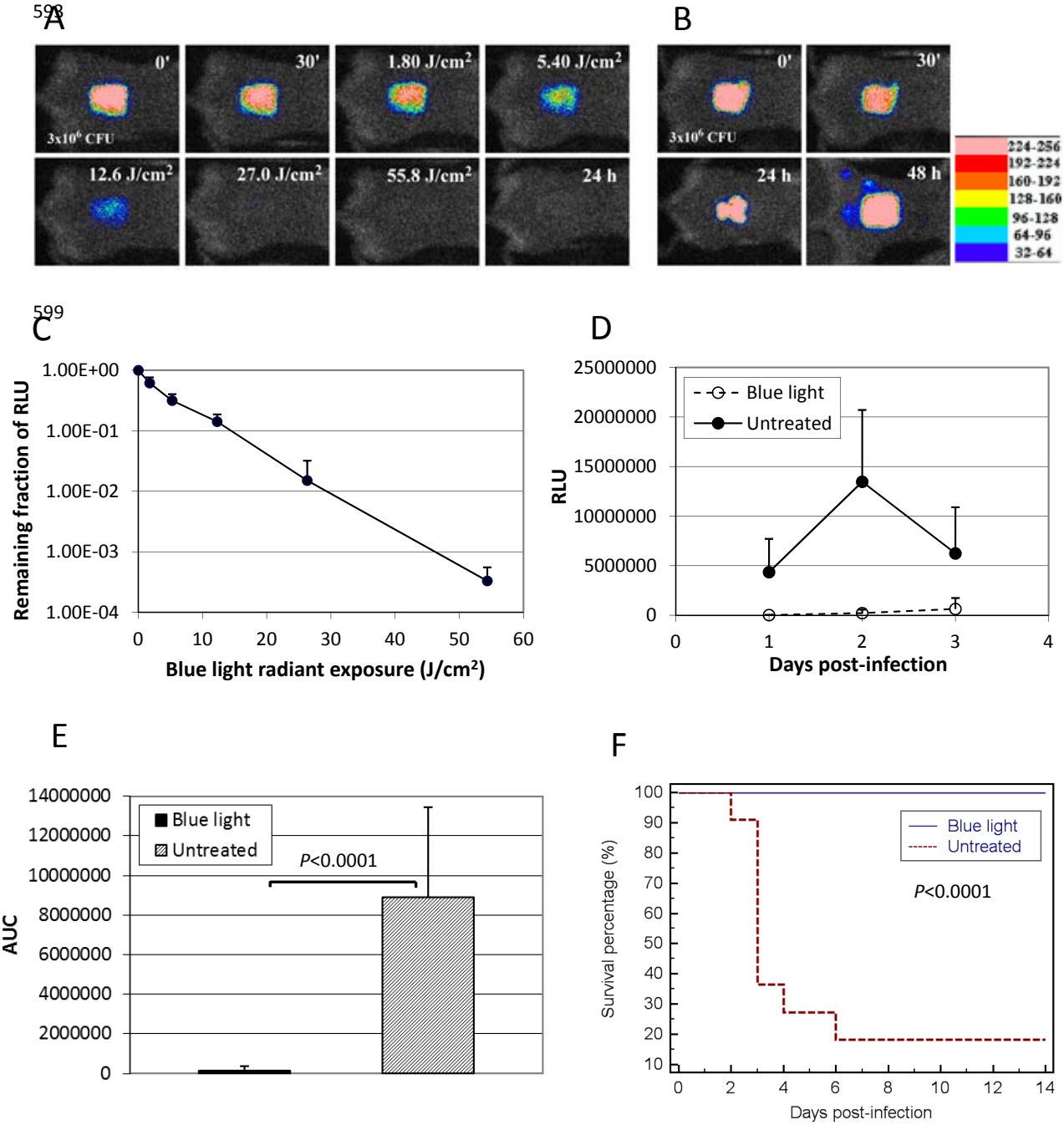

Figure 6