Published in final edited form as:

Lasers Surg Med. 2016 July ; 48(5): 562–568. doi:10.1002/lsm.22474.

Antimicrobial Blue Light Inactivation of *Pseudomonas aeruginosa* by Photo-Excitation of Endogenous Porphyrins: In Vitro and In Vivo Studies

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Abstract

Pseudomonas aeruginosa is among the most common pathogens that cause nosocomial infections and is responsible for about 10% of all hospital-acquired infections. In the present study, we investigated the potential development of tolerance of *P. aeruginosa* to antimicrobial blue light by carrying 10 successive cycles of sublethal blue light inactivation. The high-performance liquid chromatographic (HPLC) analysis was performed to identify endogenous porphyrins in *P. aeruginosa* cells. In addition, we tested the effectiveness of antimicrobial blue light in a mouse model of nonlethal skin abrasion infection by using a bioluminescent strain of *P. aeruginosa*. The results demonstrated that no tolerance was developed to antimicrobial blue light in *P. aeruginosa* after 10 cycles of sub-lethal inactivation. HPLC analysis showed that *P. aeruginosa* is capable of producing endogenous porphyrins in particularly, coproporphyrin III, which are assumed to be responsible for the photodynamic effects of blue light alone. *P. aeruginosa* infection was eradicated by antimicrobial blue light alone (48 J/cm²) without any added photosensitizer molecules in the mouse model. In conclusion, endogenous photosensitization using blue light should gain considerable attention as an effective and safe alternative antimicrobial therapy for skin infections.

Keywords

Pseudomonas aeruginosa; blue light; endogenous porphyrins; drug resistance; mouse

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Conflicts of Interest Disclosures: All authors have completed and submitted the ICMJE Form for Disclosure of Potential Conflicts of Interest and none were reported.

INTRODUCTION

Pseudomonas aeruginosa is one of the most common causes of infection, especially in patients with compromised host defenses [1]. The increased number of patients diagnosed as immuno-suppressed, coupled with those subjected to invasive medical techniques, and those at high risk of surgical site infections, has contributed to the rise in acquired *Pseudomonas* infections.

Although microbiologists have been ringing the alarm bell for years, the threat of resistance to antimicrobial drugs in healthcare settings has reached such new prominence in the popular press that the issue should be added to the list of global emergencies [2–6]. *P. aeruginosa* infections are among the most difficult to treat because effective therapeutic options are either very limited or nonexistent. There is consequently a critical need for the development of new therapeutics to tackle drug resistance [6–7]. Antimicrobial photodynamic therapy (aPDT) [8–12] has been extensively investigated as an alternative for localized infections. However, the major disadvantages of PDT are (i) the sub-optimal uptake of photosensitizers by bacteria; and (ii) the lack of selectivity of many photosensitizers for bacterial cells over host cells [13]. Recently, antimicrobial blue light therapy (aBLT) has attracted considerable attention due to its intrinsic antimicrobial effect without the involvement of exogenous photosensitizers [14–15–17]. A common hypothesis regarding the mechanism underlying the antimicrobial effect of blue light is that the natural endogenous photosensitizers, mainly porphyrins, are converted to their triplet state when exposed to light. These excited photosensitizers may generate free radicals or superoxide ions resulting from hydrogen or electron transfer (Type I), and/or they can produce singlet oxygen (Type II), all of which can react with cellular components and cause microbial cell death [8–18].

In a previous study, we investigated the effectiveness of antimicrobial blue light for treatment of lethal third degree *P. aeruginosa* burn infections in mice [14]. In the present study, we investigated the potential development of tolerance of *P. aeruginosa* to antimicrobial blue light by carrying out 10 successive cycles of sublethal blue light inactivation of *P. aeruginosa*. The high-performance liquid chromatographic (HPLC) analysis was performed to identify endogenous porphyrins in *P. aeruginosa* cells. In addition, we tested the effectiveness of antimicrobial blue light inactivation of *P. aeruginosa* in a mouse model of nonlethal skin abrasion infection by using a bioluminescent strain of *P. aeruginosa*.

MATERIALS AND METHODS

Light Source

Two different light sources both emitted blue light at a center wavelength of 415 nm with a full width at half-maximum (FWHM) of 20 nm were used. Omnilux Clear-U light-emitting diode (LED) array (Photo Therapeutics, Inc., Carlsbad, CA) (Fig. 1a) was used for the *in vitro* experiment at irradiance 20 mW/cm².

A prototype blue light LED (VielightInc) (Fig. 1b) was used for *in vivo* study, and the irradiance was 100 mW/cm². The irradiances of the blue light sources were measured using a PM100D power/energy meter (Thorlabs, Inc., Newton, NJ) and were adjusted by manipulating the distance between the LED array aperture and the target (cell culture or mouse wounds).

Bacterial Strain and Culturing

P. aeruginosa ATCC 19660 (strain 180), which causes septicemia after intraperitoneal injection [19] was used in the current study. The bioluminescent variant (strain Xen 05) carried the entire bacterial *lux* operon integrated into its chromosomes for stable luciferase expression, to be used for bioluminescent imaging (strain Xen 05 was a kind donation from Xenogen Inc., Alameda, CA) [20].

P. aeruginosa cultures were grown in brain heart infusion (BHI) medium supplemented with 50 µg/ml kanamycin in an orbital incubator (37°C, 100 rpm).

Bacterial growth was adjusted to an optical density 0.2 at 600 nm, which is equivalent to 10⁸ cells/ml. The bacterial suspension was centrifuged, washed, and resuspended in phosphate-buffered saline for experimental use.

Antimicrobial Blue Light Inactivation of *P. aeruginosa* In Vitro

Thirty-five millimeter Petri dishes were used for the photo-treatment experiment where, 3 ml *P. aeruginosa* suspension at 10⁸ CFU/ml in PBS was poured into the dishes and irradiated with a blue light LED array at an irradiance of 20 mW/cm². The irradiances were those measured on the surfaces of targets. The dishes were irradiated at room temperature. The temperatures of the cultures were measured by a thermal couple and were <30°C.

The suspension was stirred by a mini-magnetic bar (Fisher Scientific Co., Norcross, GA) during irradiation. Aliquots of 40 µl of the suspension were withdrawn at 0, 10, 20, 30, and 40 min, respectively, when 0, 12.0, 24.0, 36.0, and 48.0 J/cm² blue light had been delivered.

The aliquots were plated on square BHI agar plates, and CFU/ml were determined by serial dilutions [21]. Plates were incubated for 18–24 hours at 37°C. The experiments were repeated independently three times. Data points represent mean values±standard deviation of the three independent experiments.

Tolerance study of blue light inactivation of *P. aeruginosa*

In order to assess the possible development of tolerance to antimicrobial blue light in *P. aeruginosa*, 10 repeated cycles of sublethal inactivation of bacteria *in vitro*, followed by bacterial regrowth, were carried out. For each cycle after blue light irradiation at 36 J/cm², bacteria samples were collected and subcultured in BHI medium for the inactivation of next cycle. After 24 hours incubation from subculturing, a 3-ml bacterial suspension containing 10⁸ CFU/ml in PBS was placed into a 35-mm Petri dish.

The suspension was stirred with a miniature magnetic bar during irradiation with blue light (20 mW/cm²), and the same light exposure dose (48.0 J/cm²) were then used throughout the

10 successive cycles. Bacterial concentration (CFU/ml) was determined by serial dilution on BHI agar plates [21]. This procedure was repeated independently three times for each of the 10 cycles. Bacteria survival rates of different cycles were compared using a one-way analysis of variance test.

HPLC Analysis

Overnight broth cultures were centrifuged (rpm 3000, minute 3), and the pellet was extracted by a 0.1 M NH_4OH acetone solution (1: 9 v/v). Porphyrins from the extracts were identified by an HPLC system (agilent 1290 infinity LC system and 6430 Triple Quad MS) using a C-18 modified silica column and reverse phase system. The system was equipped with a fluorescence detector, with an excitation wavelength of 400 nm and an emission wavelength of 620 nm. Elution was performed using a gradient of acetonitrile and ammonium acetate. Porphyrin HPLC kit (Immundiagnostik AG, Bensheim) was used as a standard kit for identifying the porphyrins produced.

Mouse Skin Abrasion Wound Infection Model

Seven to eight-week-old female BALB/c mice weighing 17–21 g were purchased from Charles River Laboratories (Wilmington, MA). The mice were housed five per cage with access to food and water *ad libitum* and were maintained on a 12-hour light–dark cycle at a room temperature of 21°C and a relative humidity ranged from 30% to 70%. All animal protocols were approved by the subcommittee on Research Animal Care (IACUC) of the Massachusetts General Hospital and according to the guidelines of the National Institutes of Health (NIH).

The mice were anesthetized by intraperitoneal (i.p.) injection of a ketamine–xylazine cocktail and shaved on the dorsal surfaces. Skin abrasion wounds were made on the dorsal surfaces of mice using needles by creating 6×6 crossed scratch lines within a defined 1×1 cm area. Skin infection were generated by applying 50- μl bacterial suspension containing 4×10^7 CFU over the crossed scratches with a pipette tip [22].

Imaging System

The system consisted of an intensified charge-coupled-device (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 built inside the imaging box providing the required light for dimensional imaging. A clear image can be obtained even at extremely low-light levels by detecting and integrating individual photons one by one under the photo-counting mode. Mice were anesthetized by i.p. injections of a ketamine–xylazine cocktail before imaging. Then, the infected wounds of mice were placed on an adjustable stage directly under the camera in the specimen chamber. A grayscale background image of each infected sites was made and then followed by a photon count of the same region, whereas photon count was quantified as relative luminescence units (RLU) and was displayed in a false-color scale ranging from pink to blue.

Antimicrobial Blue Light Therapy of *P. aeruginosa* Skin Infections in Mice

The infected mice were exposed to blue light with the irradiance of 100 mW/cm² and the irradiation started at 30 minutes after bacterial inoculation. The mice were given a total light exposure of up to 48 J/cm² in aliquots with bioluminescence imaging taking place after each aliquot of light (2 and 8 minutes). The bacterial luminescence from infected mouse wounds was measured until the infections were cured.

Statistical Analyses

The inactivation rates of the cell growths curves (slopes of the survival curves) were compared for statistical analysis using a Student's *t*-test. *P* values of less than 0.05 were considered significant for all statistical analyses.

RESULTS

Phototoxicity of Blue Light to *P. aeruginosa* In Vitro

Figure 2A displays the phototoxicity of blue light to *P. aeruginosa* *in vitro*. The inhibition in the growth curves followed first-order kinetics. The inhibition of bacterial growth significantly increased with the light exposure (*P* = 0.040).

When 48 J/cm² blue light had been delivered (40 minutes of illumination at an irradiance of 20 mW/cm²), an approximately 3.54-log₁₀-cycle CFU/ml inactivation of *P. aeruginosa* was achieved.

The possible induction of tolerance of *P. aeruginosa* to blue light inactivation was measured after 10 consecutive cycles of sublethal inactivation.

Figure 2B shows the extent of bacterial inactivation by blue light under the lethal exposures dose 48 J/cm² in the 10 inactivation regrowth cycles. The tolerance study showed no evidence of the development of tolerance by *P. aeruginosa* to blue light after 10 consecutive cycles of sub-lethal inactivation. Whereas, there is no statistically significant change between the 10 consecutive cycles of sub-lethal inactivation (*P* = 0.893). Figure 2A also shows the blue light inactivation curves of *P. aeruginosa* and three cycles out of the ten (cycles 1, 6, and 9) were representative.

HPLC Analysis of Endogenous Porphyrins Extracted From *P. aeruginosa*

An accurate identification of the endogenous porphyrins was performed to identify the mechanism of the antimicrobial effects of blue light. Endogenous porphyrins that were extracted from overnight broth cultures were transferred to HPLC for reverse phase chromatography. The HPLC profiles of a mixture of standard porphyrins is shown in Figure 3A and the HPLC profiles of *P. aeruginosa* extracts is shown Figure 3B.

The chromatogram indicates that *P. aeruginosa* produces endogenous porphyrins because emission peaks were produced around 620 nm with an excitation wavelength of 400 nm. Chromatographic analyses showed that there is no difference in the HPLC profiles of both control and irradiated *P. aeruginosa*. Results also showed that the endogenous porphyrins seemed to be principally coproporphyrin.

Effect of Antimicrobial Blue Light on Mice Skin Abrasion Infected With *P. aeruginosa*

Figure 4A and B show the successive bacterial luminescence images of a representative mouse skin abrasion infected with 4×10^7 CFU of luminescent *P. aeruginosa*, with (A) and without (B) blue light therapy, respectively. The blue light was delivered at 30 minutes after bacterial inoculation. As shown in part A, bacterial luminescence was completely eliminated after 48 J/cm² blue light had been delivered (8 minutes of illumination at an irradiance of 100 mW/cm²), whereas bacterial luminescence in the untreated mouse remained almost unchanged during the same period.

Figure 4C shows the average reduction in bacterial luminescence from seven mice, each of which was exposed to blue light. The *in vivo* inactivation curve also approximately followed first-order kinetics. An average 5-log₁₀-cycle reduction of bacterial luminescence was achieved after 48 J/cm² blue light irradiation while the average bacterial luminescence from the untreated mice (seven mice) decreased by only 0.15-log₁₀-cycle during the same period ($P < 0.05$).

DISCUSSION

The current study showed the effectiveness of antimicrobial blue light at 415 nm for inactivation of *P. aeruginosa* both *in vitro* and *in vivo*. The *in vitro* study showed that *P. aeruginosa* was highly sensitive to antimicrobial blue light and over 3-log₁₀ units inactivation was achieved after a single exposure of 48 J/cm² was delivered.

On the other hand, over 3-log₁₀ units *Acinetobacter baumannii* were inactivated after a single exposure to 70.2 J/cm² blue light as shown previously [23]. Both *A. baumannii* and *P. aeruginosa* are multi-drug-resistant gram-negative strains. However, many parameters, including host factors, the bacterial burden and the virulence of individual strains, may play important roles in causing infection and the development of resistance to antimicrobial agents. Therefore, there is a difference in the exposure dose to achieve the same results [24,25].

Moreover, no evidence of tolerance development to blue light inactivation was observed after 10 consecutive cycles of sublethal inactivation of *P. aeruginosa*. This finding was in agreement with that obtained before when an *A. baumannii* strain was treated with blue light for 10 consecutive cycles of sublethal inactivation [23].

Similar to antimicrobial PDT [13], antimicrobial blue light seems to act at multiple sites within bacterial cells (structural proteins, enzymes, nucleic acids, unsaturated lipids, etc.). Therefore, it shows a low potential for the development of bacterial tolerance, as compared to conventional antibiotics, which are usually specific for a single target.

The inhibitory effect of the blue light at 415 nm toward *P. aeruginosa* suggested the presence of endogenous photosensitizers as porphyrins [14] that could play the role of endogenous photosensitizers. We investigated the presence of endogenous porphyrins by using HPLC. Results implied that *P. aeruginosa* cells produced endogenous porphyrins, and the main endogenous porphyrin was suggested to be coproporphyrin III, but further investigations

should be done in the future to support our finding. In our previous study, the emission spectra of fluorescence spectroscopy suggested that coproporphyrin III and/or uroporphyrin III could be the intra-cellular photosensitive chromophores associated with the blue light inactivation of *P. aeruginosa* [14].

The *in vivo* study showed that blue light at a 415-nm wavelength when applied 30 minutes after bacterial inoculation could effectively eradicate the *P. aeruginosa* infection in mouse skin wounds.

For aPDT, to achieve equivalent amounts of inactivation of microorganisms, higher light exposures (and higher doses of photosensitizers) were required *in vivo* than *in vitro*. Photodynamic therapy (PDT) targeted *P. aeruginosa*, by use of a polycationic photosensitizer conjugate, was monitored previously *in vitro* and *in vivo*. Results show that the maximum lethal phototoxicity *in vitro* was achieved after irradiation with 40 J/cm² of 665-nm light, in contrast, 240 J/cm² was required to produced the same effect *in vivo* [26].

This could be due to the competition between the host tissue and the microorganisms for binding to the exogenous photosensitizer, resulting in a reduced aPDT efficacy *in vivo* compared to that found *in vitro*. In contrast, inactivation of bacteria by blue light is due to photo-excitation of endogenous porphyrins in the bacterial cells. In addition, blue light targets the photosensitizing porphyrins only in the bacterial cells, whereas mammalian cells do not usually contain free porphyrins. Therefore, our results showed that almost decrease in bacterial viability *in vitro* and *in vivo* could be achieved by the same light dose, that is, 48 J/cm². In our previous study [14] on antimicrobial blue light therapy for *P. aeruginosa* burn infection, we observed that over 3-log units bacterial inactivation *in vivo* was achieved after a single exposure of blue light at 55.8 J/cm², applied 30 minutes after bacterial inoculation to the infected mouse burns. The difference in the inactivation rate between our previous study and the current one could be due to the severity of wound infection, different species of bacteria, and so on. Studies have demonstrated that, at chronic wounds, bacteria persist in adhesive, polymeric matrix biofilm communities, in which they are more resistant to antimicrobial therapy [27,28].

Finally, we can conclude that the main objectives of the present study were to assess the possible development of tolerance to antimicrobial blue light in *P. aeruginosa* and to emphasize the hypothesis about the blue light sensitivity correlated to the presence of endogen porphyrins. Our results show that the use of blue light for treating infections is of great interest because it is a non-antibiotic approach that overcomes the antimicrobial resistance drawbacks. *P. aeruginosa* produces endogenous porphyrins that could play a significant role in the antimicrobial effect of blue light. In general, our study is a preliminary research toward developing a blue light therapy as a potential alternative treatment regimen for skin infections. In the future, we plan to study how these results translated to practical situations, by developing an established infection and applying multiple sessions of blue light therapy to achieve the curative treatment.

ACKNOWLEDGMENTS

This work was supported by ASLMS; Contract grant number: BS.F04.14; NIH; Contract grant number: R21AI109172. Authors would like to thank the Binational Fulbright Commission in Egypt for their support [AY2013-2014]. MRH was supported by US NIH grant R01AI050875.

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Fig. 1. Emission Spectra of the Irradiation Sources. **(A)** represents the emission spectrum of Omnilux Clear-U light-emitting diode (LED) array and **(B)** represents the emission spectrum of blue light LED (Vielight Inc). Both light sources emitted blue light at a center wavelength of 415 nm with a full width at half-maximum (FWHM) of 20 nm.

Fig. 2.

Blue light inactivation of *Pseudomonas aeruginosa*. **A:** Data represents three cycles out of the ten (cycles 1, 6, and 9). Bars denote SDs. Ten repeated cycles of sublethal inactivation of bacteria, followed by bacterial regrowth, were carried out. Each cycle was subcultured from the bacterial survival remaining after blue light inactivation at 36 J/cm² from the previous cycle, and the same light exposure doses (0, 12.0, 24.0, 36.0, and 48.0 J/cm²) were then used throughout the 10 successive cycles. Bacterial CFU was determined by serial dilution on BHI agar plates. Data points represent mean values ± standard deviation of three independent experiments. **B:** Data represents the bacterial inactivation by blue light under the lethal exposure dose 48.0 J/cm² in the 10 inactivation regrowth cycles expressed in log₁₀ unit.

Fig. 3. HPLC profiles of porphyrins. **A:** HPLC separation of a standard mixture of porphyrins (Porphyrin HPLC kit; Bensheim). **B:** HPLC separation of the extracted porphyrins from *Pseudomonas aeruginosa*.

Fig. 4.

Bacterial luminescence images of mouse skin abrasion wound infection. **A** and **B** represents the images of mouse skin abrasion wound infected with 4×10^7 colony-forming units (CFU) of luminescent *Pseudomonas aeruginosa*, with (A) and without (B) blue light exposure. The blue light irradiance was 100 mW/cm². The blue light was delivered 30 minutes after bacterial inoculation. In part A (treated), bacterial luminescence image was taken immediately after bacterial inoculation (0 minute); 30 minutes after bacterial inoculation and just before blue light irradiation; the 32, 36, and 44 minutes images were taken immediately after 12, 24, and 48 J/cm² blue light had been delivered, and images were taken 48 hours from irradiation. In part B (Control), the images were taken at the same corresponding time points after bacterial inoculation as done in the treated group. **C**: The graph shows dose responses of mean bacterial luminescence of mouse skin abrasion wound infected with 4×10^7 CFU of *P. aeruginosa*, with ($n = 7$ mice) and without ($n = 7$ mice) blue light exposure. The blue light was delivered 30 minutes after bacterial inoculation. Bars denote SDs. RLU, relative luminescence units.