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Antimicrobial Photodynamic Inactivation and Photodynamic Therapy for Infections

Liyi Huang, Tianhong Dai, and Michael R. Hamblin

Abstract

Photodynamic therapy (PDT) was initially discovered over 100 years ago by its ability to kill microorganisms, but its use to treat infections clinically has not been much developed. However, the present relentless increase in antibiotic resistance worldwide and the emergence of strains that are resistant to all known antibiotics has stimulated research into novel antimicrobial strategies such as PDT that are thought to be unlikely to lead to the development of resistance. In this chapter we will cover the use of PDT to kill pathogenic microbial cells in vitro and describe a mouse model of localized infection and its treatment by PDT without causing excessive damage to the host tissue.

Keywords

Bacteria; fungus; microbiology; colony-forming units; *Photorhabdus luminescens* luciferase; bioluminescence imaging; mouse model of localized infection; antibiotic; wound healing

1. Introduction

It has been known since the first days of PDT early in the last century that certain microorganisms can be killed by the combination of non-toxic dyes, known as photosensitizers (PSs), and harmless visible light in vitro (1,2). Throughout the years since those times there have been additional reports of bacteria, yeasts, fungi, and viruses being killed or inactivated by various combinations of PSs and light (3,4). In the 1990s it was observed that there was a fundamental difference in susceptibility to PDT between Gram-positive and Gram-negative bacteria. It was found that in general neutral or anionic PS molecules are efficiently bound to and photodynamically inactivate Gram-positive bacterial and fungal cells, whereas Gram-negative bacterial cells are relatively resistant to these compounds (5). The high susceptibility of Gram-positive bacteria and fungi was explained by their physiology as their cytoplasmic membrane is surrounded by a relatively porous layer of peptidoglycan and lipoteichoic acid, or beta-glucan and chitin, respectively, and both these structures allow non-cationic PSs to cross (6). Then several groups of workers devised approaches that would allow PDI of Gram-negative species (5). These methods included using the polycationic peptide polymyxin B nonapeptide (5) and EDTA (7), which both increased the permeability of the Gram-negative outer membrane and allowed PSs that are normally excluded from the cell to penetrate to a location where the reactive oxygen species (ROS) generated on illumination to execute fatal damage. A second approach adopted by several groups is to use a PS molecule with an intrinsic positive charge. Wilson, Wainwright, and other groups have used the phenothiazinium salts such as toluidine blue O to carry out PDI of a large range of both Gram-positive and Gram-negative bacteria (8). The group in Italy led by Jori has used cationic porphyrins to photoinactivate Gram-negative species such as *Vibrio anguillarum* and *E. coli* (9). They found

that washing the loosely bound PS from the cells before illumination decreased the killing and explained this finding by supposing that the first dose of light on PS bound to the outside of the outer membrane causes an initial limited photodamage that then allows further penetration of the PS (10). The group in Leeds, UK, led by Brown has used cationic phthalocyanines for PDI of Gram-negative bacteria (11). They investigated *E. coli* DH5a and in particular the mechanism of uptake. They found that incubation with PPC in the dark led to increased sensitivity of the bacteria to hydrophobic but not hydrophilic antibiotics. Incubation with PPC also led to increased uptake of radiolabeled protoporphyrin that was reversed in the presence of up to 50 mM Mg^{++} ions. These observations were consistent with the uptake of PPC proceeding through the self-promoted uptake pathway (12).

Our laboratory has introduced an approach to antimicrobial PDT in which an anionic PS (chlorin e6) is covalently conjugated to polymers with basic amino groups that can bear a cationic charge at biological pH values (13). These molecular constructs can be formed between poly-L-lysine chains (pL-ce6) or polyethylenimine polymers (either linear or branched; PEI-ce6). We have previously shown that these pL-ce6 and PEI-ce6 conjugates are highly effective in mediating the PDI of both Gram-positive and Gram-negative bacteria. Their positive charges help them to bind to the negatively charged bacteria and their polycationic nature enables them to penetrate the outer membrane of Gram-negative cells by disturbing the structure of the lipopolysaccharide layers. The macromolecular nature of these conjugates gives a temporal selectivity for bacteria over mammalian cells as the latter take them up by the time-dependent process of endocytosis, while they bind rapidly to bacteria.

Although PDI of bacteria has been known for over a 100 years (14), its use to treat infections has not been much developed (8). This may be partly due to the difficulty of monitoring the effectiveness of PDT in animal models of infection. The standard method of quantifying bacterial burdens in animal models of infection involves sacrificing the animal, removing tissue and homogenizing it, and carrying out serial dilutions to provide the number of CFU/g tissue. In order to improve this process of monitoring infection after PDT, we have developed a procedure that uses bioluminescent genetically engineered bacteria and a light-sensitive imaging system to allow real-time visualization of infections (15). The use of microbial cells that have been engineered to express luciferase and the imaging of their location and cell number has streamlined and refined studies involving PDT in animal infection models as the necessity to sacrifice the animals to acquire data on the progress of the infection has essentially been eliminated. Bacterial pathogenesis appeared to be unaffected by the presence of the luciferase genes, and bioluminescence can be detected throughout the study period in animals. Furthermore, the intensity of the bioluminescence measured from the living animal correlated well with the bacterial burden subsequently determined by standard protocols. When these bacteria are treated with PDT in vitro, the loss of luminescence parallels the loss of colony-forming ability.

We have developed several models of infections in wounds and soft-tissue abscesses in mice that can be followed by bioluminescence imaging. The size and intensity of the infection can be sequentially monitored in a non-invasive fashion in individual mice in real time. When photosensitizers are introduced into the infected tissue followed by illumination with red light, a light dose-dependent loss of luminescence is seen. If the bacterium is invasive, the loss of luminescence correlates with increased survival of the mice, while animals in control groups die of sepsis within 5 days. Healing of the PDT-treated wounds is not impaired and may actually be improved.

One problem that is evident when applying PDT for microbial infection is the fact that soon after cessation of illumination, the generation of antimicrobial ROS ceases and the lifetime of these ROS in tissue is very short. Therefore there is likely to be no reason why any microbial

cell remaining alive cannot regrow without hindrance after illumination has finished. Bioluminescence imaging in fact demonstrates that in some circumstances this bacterial regrowth does occur. The hope for clinical application is that there exists some lower limit of infectious burden, so that if PDT is able to reduce the number of microbial cells beneath this limit, the host immune defense system will be able to “mop-up” the remaining microorganisms and cure will ensue. It may also be possible to repeat PDT for localized infections at defined time intervals.

The use of various animals as models for microbiological infections has been a fundamental part of infectious disease research for more than a century (16). Now, techniques of genetic alteration and manipulation have made possible the design of animals so as to be specifically applicable to the study of a myriad of diseases.

The intent for the use of animals as models of disease is to establish an infection that mimics that seen in humans. Ultimately, the goal is to seek means by which the infection can be thwarted. A key to developing an animal model is the selection of an animal whose physiology, reaction to an infection, and the nature of the infection itself all mirror as closely as possible the situation in humans. The data from animal models provide a means of indicating the potential of a treatment. Further study, involving humans, is always necessary before something such as a drug can be introduced for general use. Such human studies are subject to rigorous control.

2. Materials

The organisms described in this chapter are all classified as biosafety level two (BL2). This means that although these species are capable of causing disease in humans, they present no health hazard to laboratory personnel when standard universal precautions are taken in handling. These precautions include the use of personal protective equipment (gloves, laboratory coat, eye protection against splashes, and mask if aerosols are likely to be generated) and use of a Class 2 biosafety cabinet. All materials containing live microorganisms should be sterilized by autoclaving before disposal. Disinfectant sprays should be used to clean benches and hoods. UV light should be regularly used in biosafety cabinets to avoid contamination.

2.1. Microorganisms

1. Gram-positive bacterium *Staphylococcus aureus* strain 8325-4.
2. Gram-negative bacterium *Pseudomonas aeruginosa* strain 180.

In this report we will describe the use of *S. aureus* and *P. aeruginosa* in vitro and infections caused by *P. aeruginosa* in vivo (see Note 1).

2.2. Equipment

1. Shaking incubator
2. Stationary incubator
3. Class 2 biological safety cabinet
4. Centrifuge
5. Autoclave

¹Sources and choice of microbiological strains. Defined strains of various species of microorganisms can be obtained from culture depositories or cell banks. ATCC (Manassas, VA) is the recognized vendor in the USA and many other worldwide collections are listed at <http://www.bacterio.cict.fr/links.html>. Stable bioluminescent bacteria are available from Caliper Life Sciences: <http://www.caliperls.com/products/reagents/bioluminescent/light-producing-cells-and-microorganisms/micro-organisms/>

6. Light source
7. Power meter
8. Vortex mixer
9. Spectrophotometer (not essential but useful)

2.3. Buffers, Reagents, Solutions

1. Brain-heart infusion broth (BHI) (Fisher Scientific, Waltham, MA).
2. Phosphate-buffered saline (PBS) (Fisher Scientific, Waltham, MA). PBS is used to wash microbial cells and for serial dilutions.
3. Liquid growth media: 200 ml of distilled water and 6 g of BHI powder. All liquid media are autoclaved at 120°C for 15 min before use.
4. Solid growth media: Liquid growth media with the addition of 1.5% microbiological agar. Microbiological agar is mixed with liquid growth media before autoclaving. Solid growth media is poured into petri dishes while warm and allowed to solidify on cooling.
5. Methylene blue (MB; 3,7-bis(dimethylamino)-phenothiazinium chloride) (Sigma-Aldrich, St. Louis, MO).

Many photosensitizers (PSs) can be used to carry out PDI experiments of microorganisms (*see Note 2*). In this chapter we will describe the use of MB that can be purchased from chemical suppliers in a degree of purity suitable for antimicrobial PDI. It is soluble in distilled water and a stock solution of 5 mM can be prepared and stored in the dark at 4°C for a limited time (only a few days). PSs such as MB are of course light sensitive and unnecessary exposure to ambient light should be avoided (*see Note 3*) (*see Fig. 12.1*).

2.4. Light Source

A convenient light source consists of a non-coherent incandescent lamp capable of delivering light into a fiber-optic probe (FOP) fitted with a band-pass filter (LumaCare, Newport Beach, CA). For MB we use a 660 ± 15 nm band-pass filter that provides approximately 1 W of light that can be focused into a spot of diameter between 1.5 and 5 cm depending on the distance from the end of the FOP.

2.5. Power Meter

The intensity of the light spot was measured as follows. A power meter (model DMM 199 with 201 standard head, Coherent, Santa Clara, CA) is used to measure the irradiance (power density in mW/cm²).

2.6. Disposable Plasticware

Serial dilutions use a considerable amount of disposable plasticware.

²Antimicrobial PS. The area of PS structure is probably where the largest variation is possible. There are a relatively large number of compounds (tens) that have been reported to be antimicrobial PS in the scientific literature. Many if not most of these PS molecules are positively charged. In other words they are cationic molecules that have one or more quaternized nitrogen atoms. These molecules may be tetrapyrroles that are based on porphyrin (22,23), phthalocyanine (20,24), or chlorin (25) backbones. A second large group of compounds is composed of cationic synthetic dyes such as phenothiazinium dyes or triarylmethane dyes. In this chapter we have chosen to describe the easily available MB in order to make it possible for most people to replicate the techniques described (26).

³PS quality control. It is known that solutions of most PS are not stable indefinitely even when stored at 4°C in the dark. The dyes that are used can aggregate in aqueous solution and this is frequently not visible to the naked eye (i.e., there is no visible precipitate). However, aggregation does mean that the dyes can substantially lose effectiveness in producing photokilling of microbial cells. Visible absorption spectra can help to monitor the activity of these dyes.

1. Microcentrifuge tubes of 1.5 ml capacity (Fisher Scientific, Waltham, MA) are ideal for carrying out serial dilutions using 200 μ L yellow pipette tips.
2. Square 10 $\times$ 10 cm plastic petri dishes (Fisher Scientific, Waltham, MA) are used for incubating the serial dilutions on agar medium in order to count colonies.

2.7. In Vivo PDT of Infections

Mice are used as an animal model. Female BALB/c mice (Charles River Lab, Wilmington, MA) are obtained at 6 weeks of age when they weigh on average 20 g. Anesthesia is conveniently obtained by intraperitoneal injection of a mixture of ketamine/xylazine (100 mg/kg; 10:1 ratio). Mice are shaved on the back and the next day burns are created using brass blocks obtained from Small Parts, Inc. (Miami, FL).

3. Methods

3.1. In Vitro PDI of Microorganisms

1. Preparation of suspension of microbial cells. Prepare liquid media (brain-heart infusion broth, BHI, for bacteria) and autoclave. Prepare solid media by addition of 1.5% microbiological agar to above broth and pour into 10 $\times$ 10 cm square petri dishes. Use a sterile loop to pick a single colony from the agar plate and put into a 15-ml centrifuge tube containing 3 ml of BHI. Leave it in the shaking incubator at 37°C overnight to allow adequate aeration. In the case of fast growing species (doubling times the order of 15–30 min) a small initial inoculum will give stationary cultures overnight that generally have cell densities of the order of 10⁹ CFU/ml. It is possible to measure the approximate cell density by a simple visible absorption measurement in a spectrophotometer at 600 or 650 nm and this can be correlated to a one-off CFU determination. This will give a number such as an OD of 0.6 corresponding to a cell density of 10⁸ CFU/ml. Stationary cultures should be refreshed after a dilution of 100:1 into fresh medium for about 1 h. Cell pellets are isolated by centrifugation (13,690 $\times$ g for 5 min) and resuspended in sterile PBS to the desired density (usually 10⁸ CFU/ml) (*see* Note 4).
2. Incubation with PSs. The concentration of dye that is used and the amount of light that is delivered to some extent have a reciprocal relationship to each other. The PS that is used in this chapter, MB, has traditionally been used in higher concentrations compared to other reported antimicrobial PS. We recommend concentrations of 10 μ M for *S. aureus* and 1 mM for *P. aeruginosa* (*see* Note 5). The incubation time can be short, and it has been found that 15 min is a reasonable time. One of the most important variables is whether the cell-PS suspensions are “washed” before

⁴Microbiological culture. The possibility of contamination is an ever-present danger in microbiology. Since rich growth media are used, stray microbial cells from the environment or from the laboratory can fairly easily grow in liquid media and, if care is not taken, can completely displace the desired species over time (in some cases without the experimenter realizing). Good aseptic technique will go a long way to avoid this occurrence. In addition it is useful to be able to recognize colony morphology of specific species on agar plates. A gram stain can be used to distinguish between Gram-positive and Gram-negative bacteria.

It is sometimes necessary to distinguish between log-phase and stationary-phase cultures.

⁵In vitro PDT. It is important to realize that there is a stoichiometric relationship between the number of microbial cells and the concentration of PS in the incubation mixture. In other words if the concentration of PS is increased then the fraction of bacteria killed will go up; similarly if the number of bacteria is decreased then the fraction killed will also go up. It is important to stir bacterial suspensions as the individual cells will settle to the bottom of the tube or well during illumination and when aliquots are withdrawn, the numbers of cells will steadily increase as the cell density rises as the total volume decreases. There is an upper limit to the concentration of PS that can be used if the compound is left in solution during the illumination. This is because of the self-shielding effect. This occurs when the dye in solution absorbs a significant proportion of the light falling on the bacterial suspension and prevents sufficient light reaching the PS-loaded cells. For many PSs this selfshielding happens when the concentration reaches about 300 μ M.

If several unknown PSs are to be tested for effectiveness in antimicrobial PDT, it may be preferable to use a set of different PS concentrations (for instance 0.1, 0.3, 1, 3, 10, 30, and 100 μ M) and a single light fluence (for instance 10 J/cm² of the appropriate wavelength) instead of a single concentration and a set of fluences.

illumination or not (*see Section 3.1*, Step 5). It is usual to protect the PS–microbial cell suspensions from ambient light by covering in aluminum foil.

3. Light delivery. The best way to carry out the actual illumination is in a 24 or 48-well plate. Place 1 ml of PS-loaded bacterial suspension into a well and remove an aliquot for CFU determination at $t = 0$. The light spot can be set up to illuminate four wells equally by adjusting its diameter to 3–4 cm. At an irradiance of 100 mW/cm² a fluence (energy density) of 6 J/cm² is delivered every minute.
4. In vitro PDI experiments. We will describe several variations of how these in vitro PDI experiments are carried out because we believe that they make an important and interesting scientific point. First the suspensions of bacterial cells and dissolved MB can be washed (or not) by centrifugation and subsequent resuspension of the pellet in PBS. Centrifuge at $13,690 \times g$ for 5 min and resuspend the bacterial pellet along with tightly bound MB in the same volume of PBS. Second the variable in the PDI experiments can be the MB concentration in the incubation mixture (from 0.3 to 30 μ M as shown for *S. aureus* in Fig. 12.3a, b), and the light fluence (10 J/cm² of 660-nm light) can be kept constant. However, another way of conducting the in vitro PDI experiments is to keep the concentration of MB in the incubation mixture constant (1 mM MB in the case of *P. aeruginosa* as shown in Fig. 12.4a, b). In this case the variable is the delivered fluence that is increased up to 320 J/cm² for the difficult-to-kill *P. aeruginosa* (*see Fig. 12.2*).
5. Serial dilutions. In order to construct a light dose-response curve with survival fraction the following aliquots of microbial cell suspension are obtained. First the original cell suspension, second the suspension after incubation for 15 min with MB solution (to quantify dark toxicity of the PS), then successive aliquots of suspension removed after successive fluences of light have been delivered (for instance, 5, 10, 20, and 40 J/cm²). Each aliquot of microbial cell suspension is individually subjected to five ten-fold serial dilutions in sterile PBS. This will provide tubes with dilutions of $1\times$, $10\times$, $100\times$, $1,000\times$, $10,000\times$, and $100,000\times$. Ten μ L of each dilution is horizontally streaked on square agar plates according to the method of Jett et al. (17) (*see Note 6*).

After 24-h incubation the plates are counted. Ideally two or three rows can be counted on each plate, the results multiplied by the appropriate power of ten and averaged to give the number of CFU/ml in each aliquot of cell suspension. Survival fractions can be obtained by dividing the treatment CFU/ml by the CFU/ml in the original cell suspension (absolute control). It is possible to also perform a series of light-alone controls, but in our experience these do not show any appreciable difference from absolute control.

6. Results of in vitro PDI. Figure 12.3a, b shows PDI of the Gram-positive bacterium, *S. aureus*, with MB and red light. It is known that Gram-positive species are much easier to kill with PDI than Gram-negative species (5). When the bacterial suspension is illuminated without a wash then the bacteria are effectively eliminated (greater than six logs of killing) with MB concentrations higher than 10 μ M, combined with 10 J/cm² of 660-nm light. In sharp contrast, when the bacterial suspensions are centrifuged before illumination to remove the MB solution, the killing obtained is dramatically reduced with barely one log of bacterial reduction even at 30 μ M MB. The reason for this difference is probably that the extracellular reactive oxygen species produced also damage the bacteria and allow

⁶Serial dilutions. Bacteria have a pronounced tendency to stick together in buffers. This means that unless care is taken the actual numbers of CFU counted in the successive serial dilutions may not reflect the calculated numbers. To avoid this effect the microcentrifuge tubes should each be individually vortexed for several seconds before the next dilution is made. In the worst case small concentrations of detergents may need to be added to the PBS to encourage bacterial dissociation from clumps.

better penetration of MB into the bacterial cells and increase killing by intracellular reactive oxygen species (9). Similar findings to these have been previously reported (18).

The Gram-negative bacterial species, *P. aeruginosa*, is very much harder to kill by PDI than Gram-positive *S. aureus*. Even though all Gram-negative species are more resistant to PDI than Gram-positive species, *P. aeruginosa* in particular is one of the most resistant of the Gram-negatives. This can be seen in Figs. 12.4a, b and 12.5. Concentrations of MB much lower than 1 mM were ineffective in mediating PDI killing under any conditions. When 1 mM MB was used with a wash by centrifugation and resuspension, we obtained a light dose-dependent killing, with the relatively high fluence of 320 J/cm² being necessary to produce five logs of killing (still incomplete elimination). When the bacterial suspension was not washed from MB in solution this killing almost disappeared. The explanation for this is that the free MB in solution acted as an optical shield and prevented the light from reaching the bacteria and accomplishing the killing. In other words the better killing effect of leaving the MB in solution only applies at fairly low MB concentrations, while at higher concentrations the MB in solution quenches PDI.

3.2. In Vivo PDT of Infections in Mouse Models

All animal procedures must be approved by the Institutional Animal Care and Use Committee (IACUC) and must meet the guidelines of National Institutes of Health. The animals are housed one per cage (to prevent mice interfering with each other's wounds) and maintained on a 12-h light/dark cycle with access to food and water ad libitum. Mice receive buprenorphine (0.03 mg/kg SC BID) for 3 days after wounding for pain relief. Mice are euthanized according to protocol when their condition is assessed to be moribund.

1. Preparation of mice. The day before the creation of the burn female BALB/c mice weighing 20–25 g are shaved on the back and depilated with Nair cream (Carter-Wallace Inc, New York, NY) (Fig. 12.6a).
2. Creation of a mouse burn infection (*see* Note 8). Mice are anesthetized with an i.p. injection of ketamine/xylazine cocktail. Burn wounds are created by applying two pre-heated brass blocks ($\approx 95^{\circ}\text{C}$, $1 \times 1\text{ cm}^2$ each in area; Small Parts, Inc., Miami, FL) to the opposing sides of an elevated skinfold on the back of each mouse (Fig. 12.6b) for 10 s (Fig. 12.6c) to make a full-thickness, non-lethal, third-degree burn measuring $2 \times 1\text{ cm}$ (Fig. 12.6d) (19).

A PBS suspension (50 μL) containing 10^8 CFUs of mid-log-phase bioluminescent *P. aeruginosa* strain 180 (*see* Note 7) in sterile PBS (OD_{600} 0.6–0.8) is inoculated onto each burn from a 200 μL yellow tip pipette and evenly spread over the surface (20). The mice are imaged with the luminescence camera immediately after adding bacteria to ensure even spread of bacteria across the burn and equal bacterial loading into each burn on different mice (21) (*see* Note 9).

⁸Creation of mouse burn infection. The susceptibilities of different areas in the BALB/c mouse body to bacterial infection are variable. For example, the lower back is usually more susceptible to infection than the upper back. To ensure that the extent of infection is relatively consistent in different mice, it is recommended that the burns be made consistently on the lower half of the mouse back. When applying the bacteria solution to the mouse burns, it is also important to smear the solution evenly on the whole burned area to ensure an even extent of infection within each burn. Mouse burns can also be made by exposing the dorsal surface of mice with a template ($1 \times 2\text{ cm}^2$ opening) to hot water bath ($92\text{--}95^{\circ}\text{C}$) (29) or to the flame of an alcohol lamp (30). Bacteria can also be applied to the burn by subcutaneous injection of bacterial inoculum (31).

⁷Animal models of infection. In order to create animal models of infection only certain microbial species (and even only certain strains within a single species) can be used. Bacteria are described as having varying degrees of virulence and pathogenicity. Moreover microbes that are highly pathogenic and virulent in humans may be markedly less so in mice (and vice versa). There have even been reports of differences in pathogenicity when a certain species and strain of microbe is tested for its ability to form an infection in two different strains of mice (27,28).

3. Imaging of infections using bioluminescence. Mice are anesthetized with ketamine/xylazine and placed in a petri dish in a prone position with their backs uppermost on a laboratory stand inside the light-tight chamber 30 cm below the lens. The bioluminescence imaging setup (Hamamatsu Photonics KK, Bridgewater, NJ) consists of an intensified CCD camera mounted in a light-tight specimen chamber, fitted with a light-emitting diode, a setup that allows for a background gray-scale image of the entire mouse to be captured. In the photon-counting mode, an image of the emitted light from the bacteria is captured using an integration time of 2 min at a setting of 10 on the image intensifier control module. By use of ARGUS software (Hamamatsu), the luminescence image is presented as a false-color image superimposed on top of the gray-scale reference image. The image-processing component of the software calculated the total pixel values from the luminescence images of the infected area. The same analysis area of 1,200 pixels was used for all the wounds at all time points.

4. Addition of MB. MB is added 20–30 min after the inoculation of bacteria. MB is added as 50 μ L of a solution in PBS (1 mM MB equivalent) (Fig. 12.6e), which is added to burns that will be PDT treated or dark controls. After a further 15–30 min to allow the MB to bind to and penetrate the bacteria the mice are again imaged to quantify any dark toxicity of the MB to the bacteria.

5. Light delivery. Mice are illuminated with 660 ± 15 nm light delivered by a non-coherent light source (LumaCare, Newport Beach, CA) (Fig. 12.6f) that provides a spot on the mouse with a diameter of 3 cm and an irradiance of 100 mW/cm². The power of light is routinely measured using a power meter. Mice are given total light doses of up to 240 J/cm² in aliquots (12, 24, 48, 96, and 60 J/cm²), with bioluminescence imaging taking place after each aliquot of light (*see* Note 10). At the conclusion of the experiment, mice are allowed to recover from anesthesia in an animal warmer and resume their normal activity. There are no visible differences between any of the burns at the completion of illumination or indeed at any time during the healing process.

6. Mouse follow-up. Burns are not dressed as the bacteria tend to grow on the moist undersurface of the dressing. On each of the next 2–5 days the mice are anesthetized with a small dose of ketamine/xylazine and imaged under the same conditions (*see* Note 11). The burns are measured in two dimensions each day and the areas calculated. The strain of *P. aeruginosa* we have employed is invasive and bacterial inocula as low as 10⁵ will reliably lead to development of bacteremia and death from sepsis. Depending on the bacterial load death occurs anywhere from 2 to 10 days after infection and is preceded by a significant weight loss (10–20% of body weight). Therefore, when mice are infected with *P. aeruginosa* they are weighed each day and followed for survival in addition to their wounds being measured.

⁹Alternative mouse infection models. It is possible to use excisional wounds as a basis for bacterial infections (21,32). In some cases subcutaneous abscesses can be used as mouse models. In this case the bacterial suspension is injected under the skin and into the muscle (e.g., thigh muscle). Depending on the species of bacteria employed, temporary immune suppression of the mice using cyclophosphamide injection may be necessary to allow the bacteria to become established (33).

¹⁰PDT of burn infections. The evaporation of the solvent (PBS) during PDT can cause non-PDT killing of bacteria. To prevent this artifact, it is recommended that aliquots of 10–15 μ L PBS be added to the PDT-treated burns after each aliquot of light. Photobleaching of MB can impair the effectiveness of PDT and is a commonly encountered problem in PDT. The effect of photobleaching can be eliminated or reduced by applying MB in aliquots to the burns.

Too much light (usually >350 J/cm²) can impair the selectivity of killing of bacteria and cause non-specific host tissue damage by PDT.

¹¹Problem of regrowth. Bacterial regrowth after PDT is also a common problem. This is partially due to the fact that PDT is usually carried in the growing phase of the infection, which is usually from day 0 to day 3 after infection. For *P. aeruginosa* infection in mouse burns, bacterial regrowth can cause mortality of mice and subsequently failure of PDT. Repeated PDT (usually two to three times) or the combination of PDT with conventional antibiotics has shown to be a possible strategy to counteract the bacterial regrowth.

Blood samples are withdrawn from the orbital plexus and cultured on BHI plates for determining the presence of bacteria in the bloodstream. That can be correctly identified as the colonies are bioluminescent.

Mice are also followed for survival, body weight, and wound healing (wound area). When mice die they are dissected and organ samples (spleen, liver, and kidneys) taken for dissociation and determination of bacterial numbers and sectioned for hematoxylin–eosin staining for tissue damage.

7. Results of MB-PDT of mouse burn infection. Figure 12.7 shows the bioluminescent images obtained during the PDT treatment of a *P. aeruginosa*-infected burn in a mouse model. In order to be able to visually compare the set of images they were all collected using the most sensitive bit range on the luminescent camera. This consideration means that the images of the heaviest bacterial density contain saturated pixels. It can be seen that the bacterial bioluminescence remains fairly stable for the 20 min that the bacteria are given to adhere to the tissue of the burn and form an infection. Moreover when the MB is applied to the burn surface the loss of luminescence is minimal. After 12 J/cm² of red light has been delivered there is a small loss of signal, which becomes noticeably less after 36 J/cm² has been given. After 84, 180, and 240 J/cm² the luminescence continues its dose-dependent decrease until after the highest dose of light (240 J/cm²) there is hardly any detectable luminescence left and the loss of signal is >99% equivalent to more than two logs of bacterial killing.

This study was not designed to follow the survival of the mice, but we have published in the past (21) that PDT is capable of preventing the mice from dying of a systemic infection that develops from a localized wound infection on the back. When the PDT was able to kill 95% of the bacteria, the mice were saved from dying (*see* Note 12).

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¹²Progress course of burn infection. The main determinants of the severity of the burn infection and whether the rodents develop sepsis and die are as follows: the virulence of the particular strain, the number of bacteria applied to the burn, the size of the burn expressed as % of TBSA, whether the bacteria are applied to the surface or injected into or beneath the burn, and the length of time the heated object or liquid is in contact with the mouse skin. Without PDT, *P. aeruginosa* usually can invade through the burn into mouse bloodstream and subsequently induce fatal infection. In addition, a third-degree burn and superimposed infection can cause a deficiency of the gut barrier (34,35), which can subsequently promote bacterial translocation from the gut (36).

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Fig. 12.1. Chemical structure of methylene blue, MB, absorption spectrum and lamp emission spectrum with 660 ± 15 nm filter showing overlap.

Fig. 12.2.
Schematic cartoon illustrating an in vitro antibacterial PDT experiment with MB.

Fig. 12.3.

Killing curves of MB concentration in the incubation media versus survival fraction obtained with MB-PDT (10 J/cm² of 660-nm light) of *S. aureus*. (a) The bacteria were illuminated without a wash; (b) the bacteria were washed before illumination.

Fig. 12.4.

Killing curves of delivered fluence versus survival fraction obtained with MB-PDT (incubated at 1 mM) of *P. aeruginosa*. (a) The bacteria were illuminated without a wash; (b) the bacteria were washed before illumination.

Fig. 12.5.

Bioluminescent bacteria. *Panel a* shows the relationship between luminescence and bacterial number obtained with luminescent *P. aeruginosa* 180 over four logs of bacterial numbers as measured by a luminometer. Bacterial CFU were routinely determined by streaking out a set of serial dilutions onto agar plates. *Panel b* shows an example of bioluminescence imaging of the agar plates.

Fig. 12.6.

Procedure of PDT for mouse burn infection. (a) Mouse is shaved and depilated on the back the day before experiment. (b) A skinfold is raised on the back of anesthetized mouse. (c) Two brass blocks that have been pre-heated to 95°C in boiling water are applied to either side of the skinfold for 10 s. (d) The resulting full-thickness burn on the mouse back measures 2×1 cm. (e) Fifteen minutes after application of the bacterial suspension to the mouse burn (not shown), MB solution is applied with a pipette tip. (f) Delivery of 660 ± 15 nm light from a non-coherent light source to the MB-treated infected burn.

Fig. 12.7.

Representative successive bioluminescence images of a 10-s mouse burn infected with 10^8 *P. aeruginosa* and treated with MB-PDT.