

## Photons for Therapy: Targeted Photodynamic Therapy for Infected and Contaminated Wounds

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### ABSTRACT

**Introduction:** Battlefield wounds are frequently contaminated with pathogenic microorganisms present on uniforms and skin. Although the development of serious infections can often be prevented by antibiotics, the rise in worldwide incidence of multiply antibiotic-resistant bacteria necessitates the discovery of alternative methods. In addition, traumatic wounds and burns may contain non-perfused tissue where antibiotics cannot penetrate efficiently. The possibility also exists of the use of biological weapons with unknown antibiotic susceptibility.

**Rationale:** Previously workers have used photodynamic therapy to kill bacteria in vitro, but the use of this approach has seldom been reported in vivo in animal models of infection. We report on the use of a targeted polycationic photosensitizer conjugate between poly-L-lysine and chlorin(e6) that can penetrate the Gram (-) outer membrane together with harmless red laser light to kill *Escherichia coli* and *Pseudomonas aeruginosa* infecting excisional wounds in mice.

**Methods and Results:** We used genetically engineered luminescent bacteria that allowed the infection to be imaged in mouse wounds using a sensitive CCD camera. Wounds were infected with  $5 \times 10^6$  bacteria, followed by application of the conjugate in solution and illumination with red light. There was a light-dose dependent loss of luminescence as measured by image analysis in the wound treated with conjugate and light, not seen in control wounds. This strain of *E. coli* is non-invasive and the infection in untreated wounds spontaneously resolved in a few days and all wounds healed equally well showing the photodynamic treatment did not damage the host tissue. *P. aeruginosa* is highly invasive and mice with photosensitizer alone, light alone or untreated infected wounds all died while 90% of PDT treated mice survived. Wounds treated with PDT healed significantly better than those treated with an alternative antimicrobial (silver nitrate). We then treated mice with an infection caused by *Staphylococcus aureus* that had been allowed to grow in abscesses below the skin. Conjugate injected into the infected area together with surface illumination successfully killed the bacteria.

**Conclusions:** In view of the development of cheap portable light sources PDT may have a role to play in preventing and treating infection in combat wounds.

### 1.0 INTRODUCTION

Photodynamic therapy (PDT) is a therapy for cancer and other diseases that has received regulatory approvals for several indications in many countries [1]. Its use as a cancer treatment is based on the observation that

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certain non-toxic dyes known as photosensitizers, (PS) of which haematoporphyrin derivative (HPD, also known as Photofrin) is the best known example, accumulate preferentially in malignant tissues [2]. Therapy involves delivering visible light of the appropriate wavelength to excite the PS molecule to the excited singlet state. This excited state may then undergo intersystem crossing to the slightly lower energy but longer-lived triplet state, which may then react further by one or both of two pathways known as Type I and Type II photoprocesses, both of which require oxygen [3]. The Type I pathway involves electron transfer reactions from the PS triplet state with the participation of a substrate to produce radical ions which can then react with oxygen to produce cytotoxic species such as superoxide, hydroxyl and lipid derived radicals. The Type II pathway involves energy transfer from the PS triplet state to ground state molecular oxygen (triplet) to produce the excited state singlet oxygen, which can then oxidize many biological molecules such as proteins, nucleic acids and lipids, and lead to cytotoxicity. PDT has the advantage over other therapies of dual selectivity: not only is the PS targeted to the tumor or other lesion, but the light can also be accurately delivered to the affected tissue. Although originally developed as a cancer treatment, the most successful PDT application to date (that recently received FDA approval) has been in ophthalmology, as a treatment for age-related macular degeneration [4]. Other non-oncological applications of PDT at a less developed stage include treatments for psoriasis, arthritis, Barretts's esophagus, atherosclerosis and restenosis.

Bacterial contamination of wounds is a significant cause of morbidity, delayed wound healing and increased hospital stay [5]. In combat conditions, contamination of wounds is sometimes unavoidable, and by the time the wound is treated the bacteria may have penetrated sufficiently into the tissue to be resistant to topically applied antimicrobials. Although wound infections are treated with topical and systemic antibiotics, the rapid emergence of multi-antibiotic resistant strains of bacteria is of considerable concern [6]. In addition bacteria in traumatized or non-perfused tissues are not easily affected by oral or intravenous antibiotics [7]. Photodynamic destruction of bacteria in wounds may be an effective means of killing the bacteria while simultaneously stimulating the host immune system. In order for this treatment to be effective it is necessary to establish the factors which govern the susceptibility of various bacterial strains to photodynamic inactivation [8].

It is now accepted that Gram positive bacteria are relatively easy to kill by PDT with standard photosensitizers while Gram negative bacteria are remarkably resistant [9]. It has been shown that cationic photosensitizers, or anionic photosensitizers in the presence of exogenous naturally occurring polycationic peptides, could kill Gram negative bacteria [10-12]. It is supposed that the effect of these polycationic peptides is to permeabilize the outer membrane of gram negative bacteria allowing the sensitizer to gain access to more sensitive locations inside the bacterium.

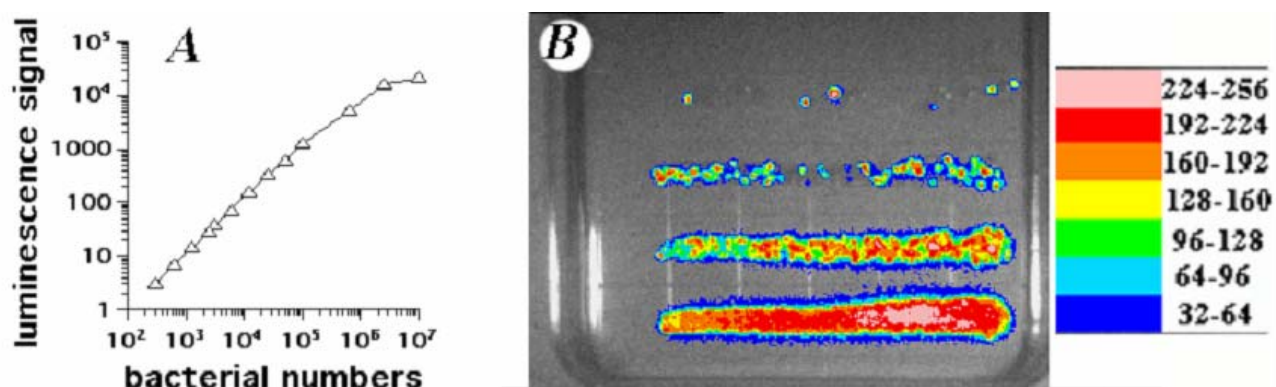
In 1997 our laboratory formed the hypothesis that by covalently conjugating a suitable PS to a pL chain a bacterial-targeted PS delivery vehicle could be constructed that would efficiently inactivate both Gram (+) and Gram (-) species. Because the resulting polycationic entity is a macromolecule, it would be taken up by mammalian cells by the time-dependent process of endocytosis, thus giving temporal selectivity for bacteria, into which the polycation could penetrate rapidly. This was demonstrated [13] by the preparation of a conjugate between one molecule of chlorin(e6) and a pL chain of 20 lysine residues that, after 1 minute incubation and illumination with red light, killed >99% of the oral pathogens Gram (+) (*Actinomyces viscosus*) and Gram (-) (*Porphyromonas gingivalis*) while sparing an oral epithelial cell line (HCPC-1). A similar construct was subsequently used by another group (composed of one ce6 molecule and a 5 amino-acid lysine chain) to kill several oral pathogens in the presence of 25% whole blood [14]. Polo and coworkers used conjugates between pL and porphycenes with a significant phototoxic activity against Gram (-) bacteria [15]. In a subsequent report [16] these authors showed that several strains responsible for periodontal disease were efficiently inactivated by visible light irradiation in the presence of porphycene-polylysine conjugates.

Repeated photosensitization of surviving cells did not induce the selection of resistant bacterial strains nor modify their sensitivity to antibiotic treatment.

We recently compared [17] the effectiveness of pL-ce6 conjugates with chain lengths of either 8 or 37 lysines attached to precisely one ce6 molecule for bacterial PDI, and found the 37-lysine conjugate was able to efficiently mediate the photodestruction of both Gram (+) and Gram (-) species, while the 8-lysine conjugate or free ce6 were only effective against Gram (+) bacteria.

## 2.0 BIOLUMINESCENCE IMAGING

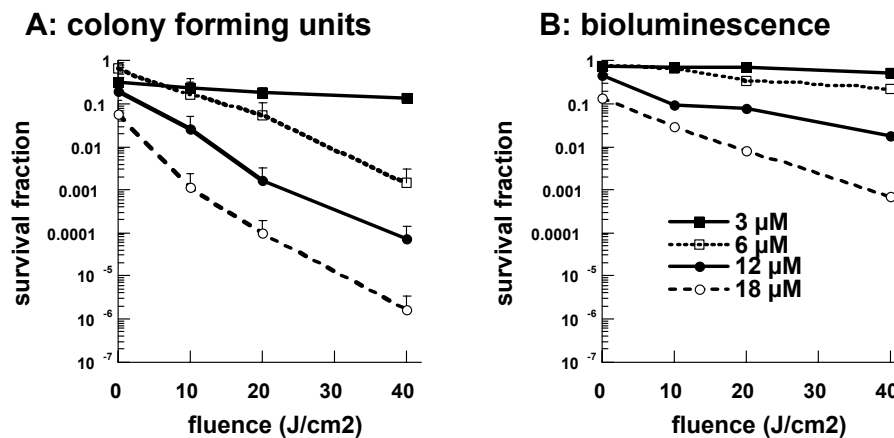
In order to explore the efficiency of PDT to kill bacteria in tissues of living animals we have developed the use of bioluminescence imaging [18-20]. We used genetically engineered bacteria that emit luminescence together with a sensitive low-light imaging camera [21-24]. These bacteria have been transfected with a plasmid containing the *Photorhabdus luminescens* lux operon (luxABCDE) that encodes for not only the luciferase enzyme, but also the biosynthetic enzymes necessary for biosynthesis of the luciferase substrate. Hence in the presence of flavin mononucleotide from the bacterium and external oxygen these bacteria will glow in the dark. The rate of luciferase enzyme turnover in the presence of substrate allows for real-time measurements, and the enzyme is active at the body temperature of mammals. An image captured by the camera of a living mouse gives information about the intensity and spatial spread of the infection, and each animal can be followed longitudinally dramatically reducing the numbers of animals needed to study treatment of infections. This method is an improvement on the traditional use of survival or body fluid sampling and subsequent plating and colony counting. Rocchetta et al studied the growth of bioluminescent *E. coli* in the neutropenic mouse-thigh abscess model of infection [25]. They found that the number of CFU extracted from the thighs of sacrificed animals closely paralleled the luminescence signal at several timepoints after inoculation and during the period of action of antibiotics.



**Figure 1A shows the relationship between luminescence and bacterial number obtained with luminescent *E coli* DH5a over four logs of bacterial numbers as measured by tube luminometer. Bacterial cfu were routinely determined by streaking out a set of serial dilutions onto agar plates. Figure 1B shows an example of bioluminescence imaging of the agar plates.**

We first ascertained that the luminescence signal measured in the luminometer was linearly proportional to bacterial CFU (as determined by serial dilution and plating) from  $10^3$ - $10^7$  organisms (Figure 1A). The signal saturated at large bacterial numbers i.e.  $> 10^7$ . In addition to counting the colonies it is possible to image the plates using the luminescence camera and this is shown in Fig 1B.

We then carried out in vitro PDI experiments in order to verify that killing the bacteria as demonstrated by loss of colony forming units correlated with loss of luminescence. The loss of viability curves as measured by CFU and by loss of luminescence, as a function of light-dose, for bacteria incubated with 3, 6, 12 and 18  $\mu\text{M}$  pL-ce6 conjugate are shown in Figs 2A and 2B. The CFU assay had a limit of sensitivity of six orders of magnitude in reduction of viability, while the bioluminescence assay had a limit of three orders of magnitude. Loss of luminescence showed the same dose-response curves as loss of CFU but the absolute reductions were 1 to 3 logs less. The reasons for this are twofold. The limits of sensitivity of the luminescence assay with the plate reader is a 3 log reduction in signal, while the CFU assay can measure a 6 log reduction in viability. Secondly it appears that the cytotoxic insult to the bacteria causes loss of viability more readily than loss of luminescence. The mechanism by which luminescence decreases after photoinactivation (PDI) is uncertain, but may be due to exhaustion of FMN supplies from the bacteria (needed for the luciferase enzyme to make luminescence) and which cannot be replenished if the cells are fatally damaged.



**Figure 2. Phototoxicity as determined by A, CFU; and B, luminescence assays. Bacteria were incubated with stated concentration of conjugate as above, then washed and illuminated with stated fluence of 665-nm light with removal of aliquots of bacterial suspension at intervals (100, 200 and 400 seconds respectively) and serial dilution and plating or luminescence measurement in 96-well plates. Data points are means of triplicate determinations and two separate experiments and bars are SD**

### 3.0 PDT OF *E. COLI* INFECTED EXCISIONAL WOUNDS IN MICE.

We initially sought to establish the animal model of infection by inoculating *E coli* into an excisional wound on the mouse [18]. Five million cfu from a mid-log culture in 50  $\mu\text{l}$  gave a sufficiently bright luminescence signal from the wound to allow at least two logs of signal reduction to be accurately followed. When this bacterial inoculum was placed into a wound (12.5 X 8 mm) made on the back of a freshly euthanized mouse, the luminescence started to fade rapidly and was totally gone by the time the 50  $\mu\text{l}$  inoculum had dried (< 60 minutes) (data not shown). Untreated infected wounds in living mice showed only a slight loss of luminescence over a period of four hours. We interpret these findings to mean that the living mouse wound



provides nutrients and moisture to the bacteria, and thus is a reasonable model of wound infection. The next day however, control infected wounds in living mice had lost on average 90% of the original luminescence signal, but with considerable inter-animal variability (data not shown).

Since the wound infection with *E coli* DH5a was found to be self-limiting, i.e. this particular strain of *E coli* is non-invasive [26], it allowed the use of each mouse as its own control to follow wound healing with four wounds per mouse. The effect of topical application of pL-ce6 conjugate and successive applications of 660-nm light is presented in a series of overlaid luminescence (false-color) and gray-scale reference images (Fig. 3). These data were obtained from a mouse in which bacteria were inoculated in all wounds, conjugate was added to wounds 1 and 4, and wounds 3 and 4 were illuminated with red light. Therefore wound 1 was the dark control with conjugate, wound 2 was the absolute control, wound 3 was the light alone control, and wound 4 was PDT treated. Topical application of a targeted polycationic PS conjugate followed by illumination led to a 99% reduction in luminescence as measured by imaging software on the luminescence images. There was a semi-logarithmic light dose-dependent reduction in luminescence from the PDT treated wound not seen with any of the control wounds, as would be expected from a standard PDT experiment (Fig. 4). There was an initial modest decrease in luminescence from wounds that received conjugate without light due to the dark toxicity of the conjugate, but the luminescence did not decrease further during the course of the experiment.

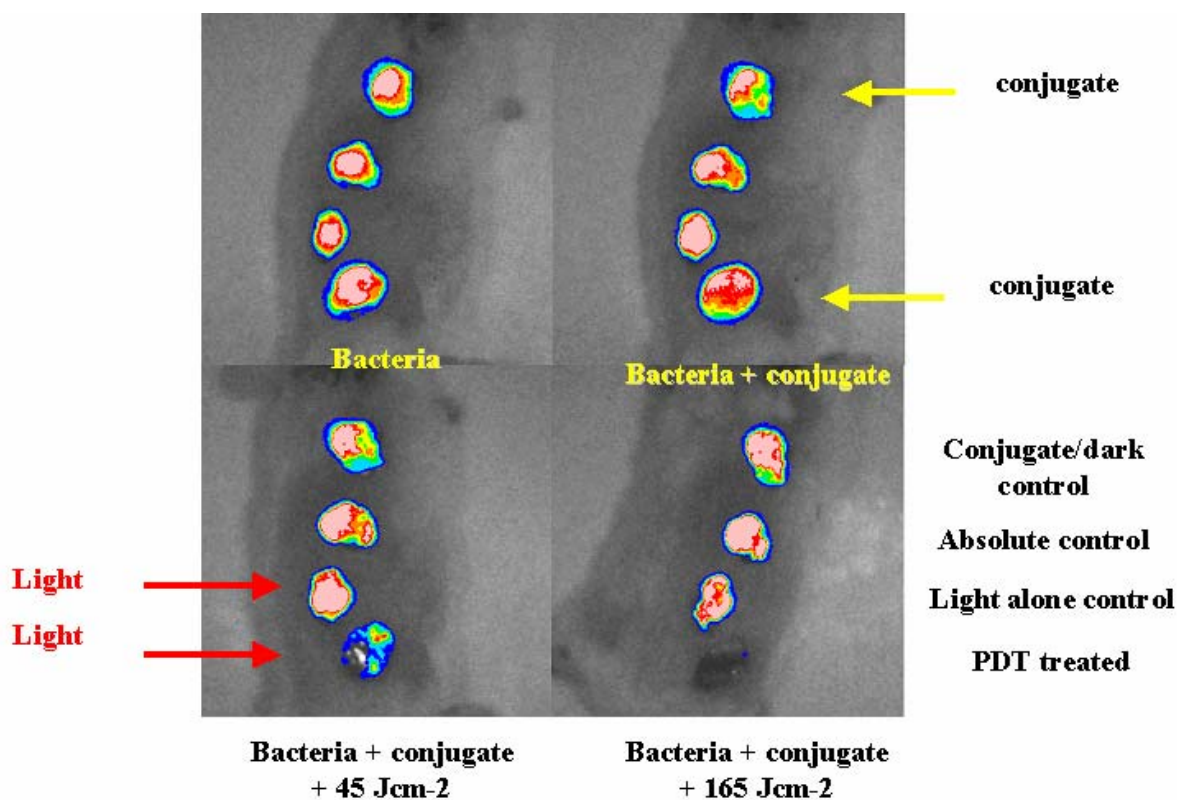


Figure 3. Successive overlaid luminescence false color images and monochrome LED images of a mouse with four excisional wounds infected with equal numbers of *E coli* (A). Wounds 1 (nearest tail) and 4 (nearest head) received topical application of conjugate (B). Wounds 1 and 2 (two nearest tail) were then illuminated with successive fluences (45 - 165 Jcm<sup>-2</sup>) of 665-nm light (C-D).

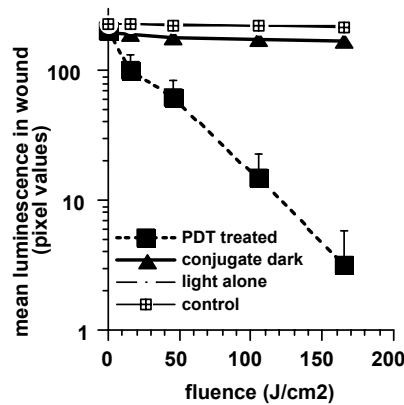


Figure 4. Mean pixel values of luminescence signals from defined areas of wounds measuring 1200 pixels determined by image analysis. Data points are means of values from the corresponding wound on six mice per group and bars are SD.

**3.1 Wound healing.** The assumption that considerable amounts of conjugate bound to the tissue in the wound suggested that illumination might have caused damage to the host cells, blood vessels or extracellular matrix in the wound. However we observed that PDT of infected wounds did not lead to any inhibition of wound healing as seen in Fig 5. There was an indication that the PDT treated wounds actually healed somewhat faster relative to the other control wounds but this was not statistically significant. The lack of host tissue phototoxicity may have been due to the necessity for a macromolecular species such as pL-ce6 (molecular weight approximately 18,500) to be taken up into mammalian cells by the time dependent process of endocytosis. In the present experiments the absence of wound healing inhibition may be explained by a combination of the topical delivery method together with the large conjugate size and the relatively short incubation time. The fact that treated wounds healed as well as control wounds suggests that PDI may have advantages over topical anti-microbial products that have been reported to cause tissue damage or have other undesirable side-effects.

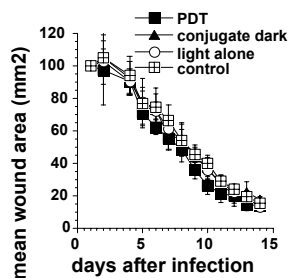
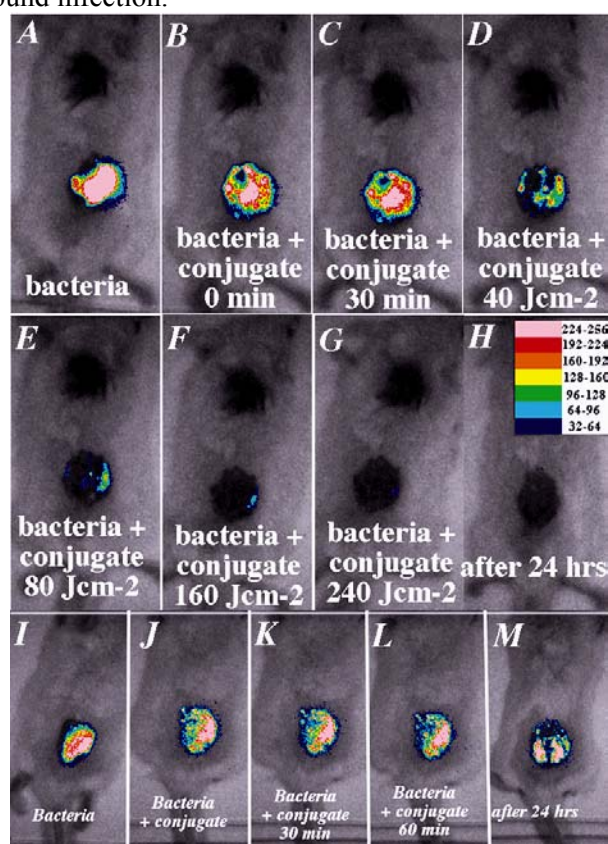


Figure 5. Mean areas of wounds from six mice per group treated as above. Wounds were measured daily in two dimensions and areas calculated. Bars are SD

#### 4.0 PDT OF LETHAL *PSEUDOMONAS AERUGINOSA* WOUND INFECTIONS IN MICE

The previous experiments were a proof-of principle study using a relatively non-pathogenic strain of *E. coli*, DH5alpha that lacks virulence factors necessary to cause invasive infections [26]. In order to test PDT in a more clinically relevant infection model, we used optical techniques (bioluminescence imaging and targeted PDT) to monitor and cure mice of an otherwise fatal *P. aeruginosa* wound infection [19]. Mice with wounds infected with  $5 \times 10^6$  CFU of *P. aeruginosa* quickly developed an illness consistent with systemic sepsis. They lost weight, had ruffled coats, and developed progressive inactivity leading to a moribund condition and death occurred between 24-60 hours after infection. When the effect of varying the initial bacterial inoculum was studied, it was found that the LD50 was approximately 200,000 CFU. Mice infected with the lower numbers of bacteria that did not die suffered the same infection as the mice that died, but in a less severe form and recovered between days 6-8. We nevertheless decided to use a bacterial challenge for the PDT experiments that was 25 times higher than the LD50 to provide a robust test of the ability of the technology to prevent death from a fatal wound infection.

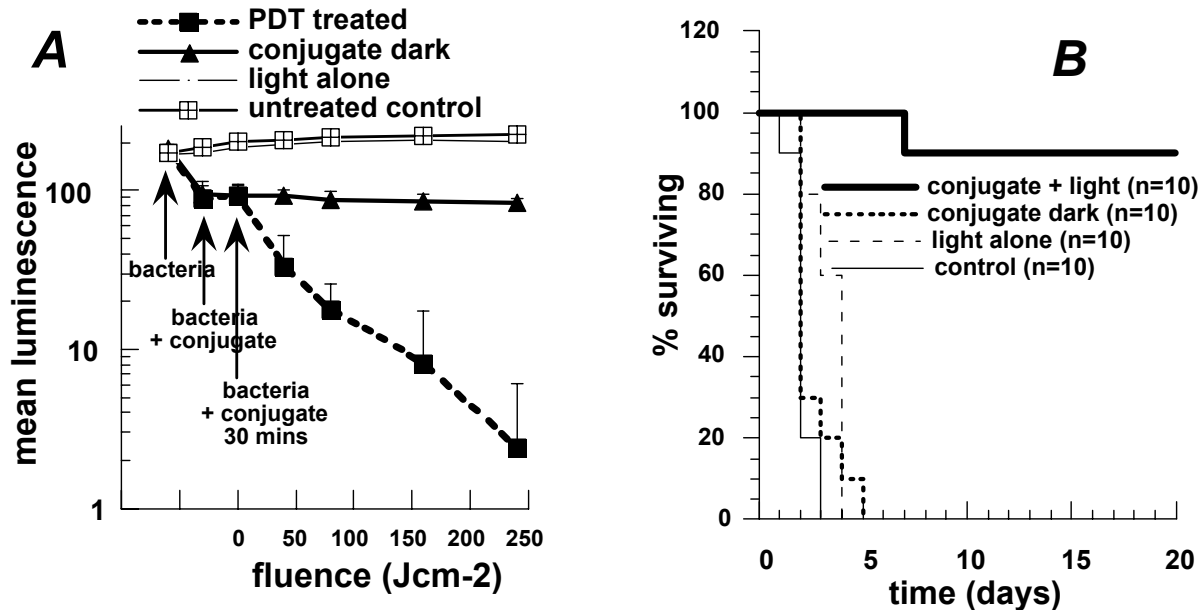


**Figure 6. Successive overlaid luminescence false color images and monochrome LED images of mice bearing an excisional wound infected with  $5 \times 10^6$  luminescent *P. aeruginosa*. (Panels A-H). A representative mouse treated with pL-ce6 conjugate and increasing doses of light. (Panels I-M). A representative mouse treated with pL-ce6 and kept in the dark.**

Mice were given a single dorsal excisional wound with an area of 100 mm<sup>2</sup>, infected with  $5 \times 10^6$  CFU of *P. aeruginosa* suspended in 50  $\mu$ L of PBS. This inoculum gave a sufficiently bright luminescence signal from



the wound to allow at least two logs of signal reduction to be accurately followed. The bacteria quickly attached to the tissue surface of the panniculus carnosus as evidenced by the failure of an attempt to wash them off by irrigation with saline 30-min after infection, and as quantitated by luminescence imaging. The pL-ce6 conjugate was added as 50- $\mu$ L of a 200- $\mu$ M ce6 equivalent concentration as preliminary experiments had shown lower concentrations to be less effective. This volume was sufficient to spread evenly throughout the surface of the wound and was retained by the edges of the wound to prevent the liquid running off. It was necessary to give the conjugate at least 30-min to bind to, and penetrate the bacteria in order to see effective loss of luminescence after illumination with 660-nm light. As can be seen from a set of luminescence images from a representative mouse shown in Fig 6A-6G, PDT produced a fluence-dependent loss of luminescence until only a trace remained after 240 J/cm<sup>2</sup> had been delivered. When the mouse was imaged the next day all traces of luminescence had gone (panel 6H). There was a drop in luminescence seen shortly after applying the conjugate in the dark (Fig 6B, 6J), but this did not decrease further after 30 min incubation (Fig 6C, 6K) or indeed after 60 min incubation (Fig 2L, approximately equal to the time for illumination of the PDT wounds). Infected wounds left untreated or treated with illumination alone showed a rise in luminescence signal (up to 2-fold, Fig 7A) presumably due to growth of the bacteria in the nutrient rich medium of the wound. There was significant luminescence present in control wounds until death occurred 2-4 days later (Fig 6M). The mean luminescence values determined from the infections in the wounds of all the mice in the four groups were calculated using the ARGUS software. The resulting curves are plotted in Fig 7A. The PDT treated group shows a semi-logarithmic relationship between bacterial luminescence and delivered fluence, until 99% of the luminescence has disappeared after 240 J/cm<sup>2</sup>. There is a significant difference between the luminescence found from the conjugate in the dark group, compared with that in the light alone and untreated control groups. This is due to two factors; firstly to a degree of dark toxicity of the conjugate towards *P. aeruginosa*, and secondly to the ability of the bacteria in the untreated and light alone control wounds to continue to grow.



**Figure 7A.** Mean pixel values of luminescence signals from defined areas measuring 1200 pixels covering infected wounds determined by image analysis. The four groups comprise untreated control, light alone control, dark conjugate control, and PDT treated. Data points are means of values from the wounds on ten mice per group and bars are SD. **Figure 7B.** Kaplan-Meier survival plot for the four groups of mice described in Figure 7A.

All the mice in the three control groups (untreated, light alone and dark conjugate) died within a period of five days infection. By contrast 90% of the mice treated with PDT survived as seen in Fig 7B. These mice appeared to suffer from some symptoms of bacterial infection (weight loss, ruffled fur and inactivity) similar to those mice who received a sub-lethal dose of bacteria described above. They recovered quickly however and by five days after infection were regaining weight and moving normally.

## **5.0 PDT OF ESTABLISHED SOFT-TISSUE INFECTIONS**

The previous experiments were carried on animals whose wounds were recently contaminated with relatively large numbers of CFU. It is unlikely that patients or combat personnel would present for treatment under these circumstances. A more realistic and clinically relevant model would consist of inoculation of a smaller number of bacteria and then allowing the infection to grow and become established in tissue over time. A second major consideration was that the previous experiments used an infection model where the bacteria were relatively near the surface of the tissue in an excisional wound. In real life the bacteria could be beneath the surface of the skin or tissue either because they had already invaded or because they were on skin or clothing that had been forcibly introduced into a penetrating wound such as those caused by gunshot or shrapnel. Since the penetration of visible light (even red light) into tissue is limited we asked whether PDT could be used to treat an infection where the bacteria had been allowed to multiply several hundred-fold and were some distance beneath the skin surface.

We used the injection of 1 million log-phase *S. aureus* CFU (mouse pathogenic strain 8325-4) into the mouse thigh muscle (2-mm deep). The mice had previously been rendered temporarily neutropenic by pre-treatment with cyclophosphamide. We used a model in which the mice developed two equivalent infections, one in each hind thigh in order to allow each mouse to have a PDT treated infection and a control untreated infection and act as its own control [20]. Twenty-four hours after infection the bioluminescence had increased dramatically as the bacteria had multiplied within the infected tissue (Figure 8). The pL-ce6 conjugate (50- $\mu$ L) at a concentration of 1-mM ce6 equivalent was injected into the infected area resulting in a visible green coloration noticeable beneath the skin. This allowed a judgment to be made about the uniformity of the PS distribution within the infection. Although these mice were neutropenic, and the infection did not accumulate the considerable quantities of pus expected from immunocompetent mice, there was still some matter present in the infection. We had previously suspected that the conjugate might diffuse relatively rapidly through the tissue, however this did not prove to be the case. The green coloration remained in place for some time (several hours) especially in unilluminated mice. Light (660-nm) was delivered to the infection as a spot on the skin about 8-mm in diameter centered on the infected area,

There was a slight reduction in bacterial bioluminescence observed immediately after the conjugate was injected into the infection. Luminescence was further reduced after the 30-minute incubation period in the dark. When illumination was commenced there was a light-dose dependent decrease in luminescence after each 40 J/cm<sup>2</sup> increment of red light (Figs 8). A typical mouse treated with injection of conjugate into the right infected thigh, followed by illumination of this thigh as described previously is shown in Fig 8, together with the mean bioluminescence values from both legs of 5 mice in Fig 9. After 160 J/cm<sup>2</sup> had been delivered the bioluminescence of the treated infected legs had been reduced by >99% compared to the untreated contralateral legs. However 4 out of 5 of these treated legs suffered a recurrence of the bioluminescence on succeeding days (data not shown).

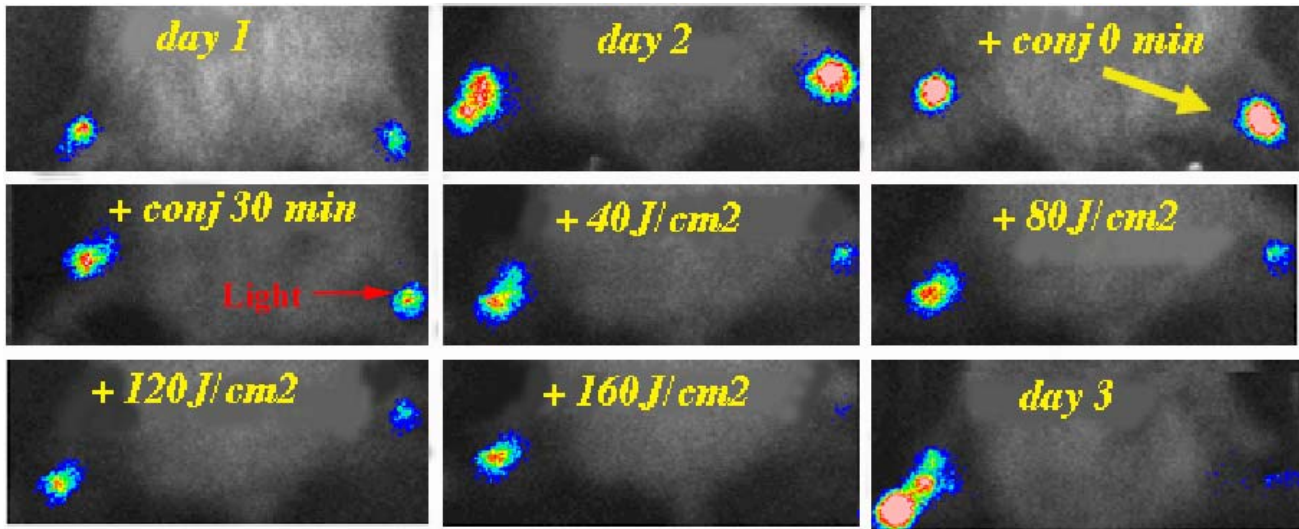


Figure 8. Series of bioluminescence images (captured at a bit range of 2-4) from a neutropenic mouse infected on day 1 in both thighs, and treated on day 2 with injection of pL-ce6 into the right thigh, followed after 30 min by illumination of the right thigh with 660-nm light at a fluence rate of 100 mW/cm<sup>2</sup>.

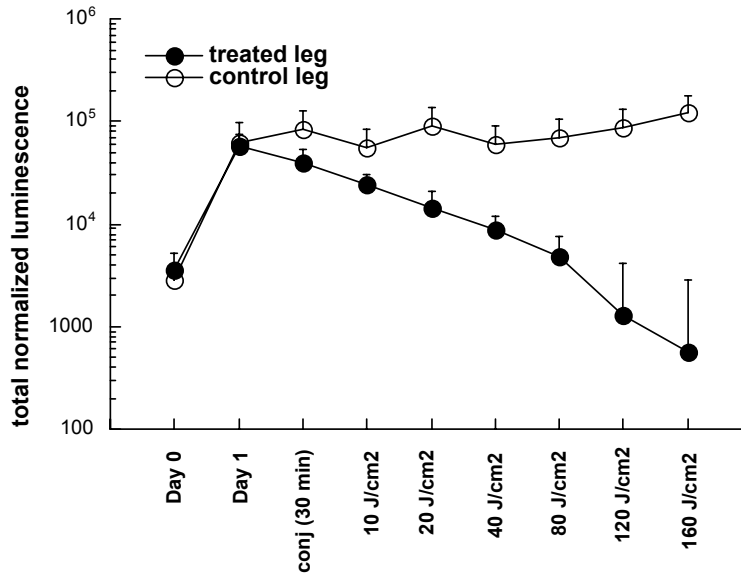


Figure 9. Mean total normalized bioluminescence values from left (untreated) and right (PDT treated) thighs of 5 mice infected in both thighs. Bars are SEM.

## 6.0. CONCLUSIONS AND FUTURE WORK

The use of a polycationic conjugate between the PS ce6 and a poly-L-lysine chain delivers the PS in an efficient manner to bacteria infecting wounds and tissue, and in addition, the delivery of light a short time after the PS gives temporal selectivity for the bacteria over the host cells as the conjugate binds quickly to bacteria but only slowly to host cells. The use of stably transduced bioluminescent bacteria together with a low-light imaging camera provides an efficient and versatile method of monitoring the progress of the infection in experimental animals in real time and in a longitudinal fashion allows response to therapy to be followed. Due to the widespread occurrence of antibiotic resistance amongst pathogenic bacteria and the relatively slow response to antibiotics when bacteria are infecting traumatized tissue, alternative methods of killing bacteria in wounds are being sought. The recent development of portable and low-cost light sources such as light emitting diodes that are battery-powered may make possible the deployment of systems that can be used for wound decontamination. Our data demonstrating that PDT can also be used for established infections further extends its possible applications.

In future work we are studying PDT for infections in thermal and chemical burns and in other medically and militarily important localized infections. These include fungal and parasitic infections of the skin (dermatophytosis and Leishmaniasis), bacterial keratitis, sinusitis, periodontitis, gastric *Helicobacter pylori* infection, bacterial cystitis etc. The local delivery of the PS and the ability of fiber optics to deliver light anywhere within the body suggest that PDT can treat infections with many hollow organs. We are also working on second generation PS-conjugates with even higher activities and selectivities for bacteria than the pL-ce6 conjugates described.

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