Journal of Antimicrobial Chemotherapy (2006) **57**, 1227–1230
doi:10.1093/jac/dkl096
Advance Access publication 20 March 2006

JAC

Induction of lethal photosensitization in biofilms using a confocal scanning laser as the excitation source

C. K. Hope[1,2]* and M. Wilson[2]

[1]School of Dental Sciences, The University of Liverpool, Liverpool, UK; [2]Division of Microbial Diseases, UCL Eastman Dental Institute, University College London, London, UK

Received 12 January 2006; returned 31 January 2006; revised 1 March 2006; accepted 2 March 2006

***Objectives*: To induce lethal photosensitization in biofilms of *Streptococcus pyogenes* using the scanning laser in a confocal microscope to photoactivate Sn (IV) chlorin e6 (SnCe6) while simultaneously measuring changes in cell vitality using fluorescent indicators of membrane integrity.**

***Methods*: Biofilms of *S. pyogenes* were immersed in a solution of 50 mg/L (70.28 μM) SnCe6 and scanned with the 488 nm argon and 543 nm HeNe lasers in a confocal microscope. Changes in membrane permeability were quantified using image analysis tools.**

***Results*: Cell permeability increased in biofilms of *S. pyogenes* after successive scanning/exposure cycles in the presence of SnCe6.**

***Conclusions*: Cell death was induced in biofilms of *S. pyogenes* by the photosensitizer SnCe6 on exposure to the scanning laser emissions of a confocal microscope. The simultaneous recording of cell death demonstrates the real-time evaluation of a light-activated antimicrobial compound against a biofilm.**

Keywords: photodynamic therapy, *Streptococcus pyogenes*, Sn (IV) chlorin e6

Introduction

With ever-increasing levels of antibiotic resistance, light-activated antimicrobial agents (photosensitizers) are becoming an attractive alternative to conventional antibiotics.[1] When a photosensitizer is excited by light photons it can transfer energy to molecular oxygen, a process which in turn generates singlet oxygen ($^1O_2$), a highly reactive cytotoxic species. Photodynamic therapy (PDT, the use of photosensitizers and light in the treatment of disease) has two main advantages over conventional antibiotic treatments. First, the bactericidal activity is confined to areas which have been treated using the photosensitizer and light—avoiding disruption of the indigenous microbiota at sites distal to the infected area.[2] Second, the development of resistance to $^1O_2$ by bacteria is unlikely due to its non-specific mode of action.[3]

Conventional assays for determining the effects of PDT typically involve exposing bacteria to light in the presence of a photosensitizer and then counting the number of viable bacteria remaining in the samples afterwards. The control experiments which are normally incorporated into these studies include the use of the photosensitizer without light, light without the photosensitizer and neither light nor the photosensitizer. These assays therefore involve two culturing steps: growing the target bacteria to produce a suitable sample followed by the recovery and enumeration of bacteria post-exposure by re-growth on a solid growth medium. Using such techniques, lethal photosensitization has been shown to be effective in killing oral bacteria in liquid cultures[4,5] or those growing as biofilms.[6]

The effects of PDT could be evaluated more efficiently, by modifying a technique in which biofilms of bacteria are treated with *Bac*Light™ LIVE/DEAD stains and then exposed to an antimicrobial compound.[7] Confocal laser scanning microscopy (CLSM) was used to record the changes in cell membrane integrity (i.e. cell death) during this exposure, in real-time. This process could be adapted to determine whether the laser light emitted during CLSM can photoactivate Sn (IV) chlorin e6 (SnCe6) in biofilms of *Streptococcus pyogenes*, while simultaneously recording changes in cell membrane integrity. This would allow the evaluation of photosensitizers without the need for a recovery/enumeration step.

*Correspondence address. School of Dental Sciences, The University of Liverpool, Edwards Building, Daulby Street, Liverpool L69 3GN, UK. Tel: +44-151-706-5252; Fax: +44-151-706-5809; E-mail: c.hope@liv.ac.uk

Methods

S. pyogenes ATCC 12202 was grown overnight in nutrient broth (Oxoid, Basingstoke, UK) at 37°C. Subsequently, 100 µL of the culture was spread over a sterile 0.45 µm pore-size nitrocellulose filter-membrane (Sterilin, Hounslow, UK) which had been laid onto a blood agar (Oxoid) plate. This plate was incubated at 37°C in a 5% CO_2 atmosphere overnight, resulting in a biofilm on the surface of the membrane.

A biofilm-laden membrane was removed from the agar plate and placed on a 50 mm diameter cell-culture dish, and 6 mL of a PBS (Oxoid)-based viewing solution containing 0.5 µL/mL of each of the *Bac*Light stains (Molecular Probes, Eugene, OR) was poured over the biofilm. This was then examined using an Olympus BX-51 upright microscope equipped with a Bio-Rad Radiance 2100 confocal laser-scanning head. The laser emissions used were argon (488 nm) and HeNe (543 nm) to excite the LIVE and DEAD stains, respectively. Prior to the treatment, *S. pyogenes* biofilms contained mostly viable cells with only a small number of individual non-viable cells apparent in the deeper layers of the biofilm (data not shown). The Ar laser power was set at 50% of that available, which proved to be a reasonable compromise between achieving photoactivation and photo-bleaching. The light dose incident on the biofilm samples was subsequently measured using a photodiode-type laser power meter (DD-2A, Ophir Optronics, Jerusalem, Israel). SnCe6 (Frontier Scientific, Carnforth, UK) stock solution (100 µL) was added to the viewing solution, yielding a final concentration of 50 mg/L (70.28 µM). A series of CLSM time-lapse scans were taken through a single optical-section of the biofilm, each of which lasted for 8 s (based on a software estimate derived from the user settings; 1024 × 1024 pixels, 500 lines/s, Kalman averaging filter set at 2 scans) and repeated on a 15 s cycle for 5 min. The total exposure time was 160 s during the 5 min sequence, yielding an energy dose of 1.05 J and an energy density of 1.17 kJ/cm. The biofilm was then allowed to rest for 10 min in order to recover from photobleaching and allow any changes in cell membrane integrity to be expressed via the fluorescent stains. This sequence was repeated three times (Figure 1). The images were analysed to reveal the average pixel brightness values, which were calculated within a user-defined region of interest drawn around the pixels occupied by the bulk of the biofilm, to avoid 'dilution' of the brightness measurements caused by unoccupied pixels.

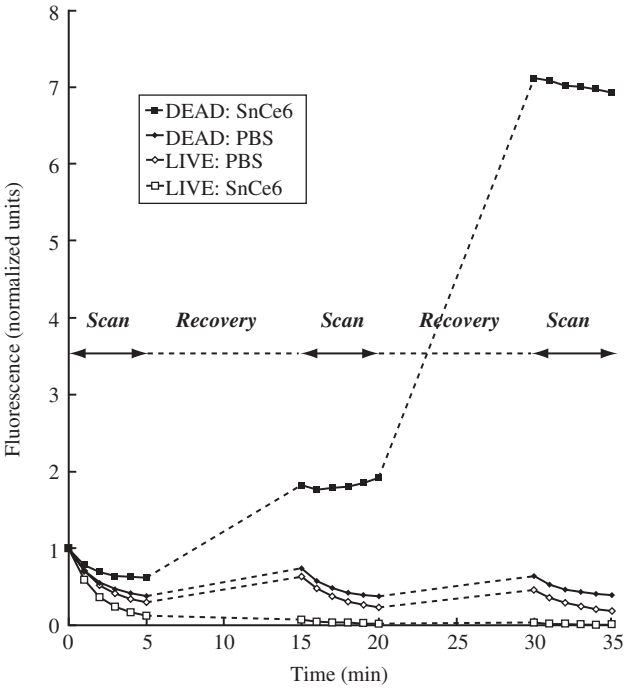

Figure 1. Simultaneous lethal photosensitization and monitoring of cell membrane integrity in a biofilm of *S. pyogenes* after repeated scanning/exposure cycles (0–5, 15–20 and 30–35 min) using a confocal laser microscope incorporating intermediate recovery periods (5–10 and 20–30 min). Filled squares, DEAD-SnCe6 (50 mg/L); filled diamonds, DEAD-PBS; open diamonds, LIVE-PBS; open squares, LIVE-SnCe6 (50 mg/L). The units of image fluorescence were normalized to their initial value. Primary laser (488 nm) energy dose, 1.05 J; energy density, 1.17 kJ/cm^2.

Results

Based upon the volume of confocal image stacks, the biofilms were at least 90 µm thick.

In the control samples in PBS without SnCe6, the fluorescence values of both the LIVE and DEAD stains decreased during the 5 min scanning/exposure element of the sequence due to photobleaching of the dyes. By the end of the recovery time, the fluorescence values of the DEAD stain increased slightly while the LIVE stain remained static—however, the general trend over the time course was decreasing fluorescence due to photobleaching.

Samples which contained 50 mg/L SnCe6 showed less photobleaching compared with PBS controls during the initial exposure (Figure 2), probably due to the light shielding effects of the photosensitizer.[8] After the first recovery period (between 5 and 15 min), the amount of fluorescence due to the DEAD stain increased significantly while that due to the LIVE stain decreased. This effect was more dramatic following the second recovery period (between 20 and 30 min).

Since light is essential for the function of fluorescent stains, it was not possible to conduct a control without light; however, an experiment using the Ar laser at 2% power (energy dose 25.12 mJ; energy density 8.32 J/cm^2) resulted in a maximum DEAD fluorescence of 1.291 units, compared with 7.119 units at 50% power (1.05 J; 1.17 kJ/cm^2)—indicative of a light dose response. The HeNe laser was fixed at 20% power (25.12 mJ; 27.91 J/cm^2) in all the experiments, a setting which yielded 2.4% of the energy dose of the 'primary' 488 nm Ar laser. Other studies have already shown that SnCe6 has no intrinsic antibacterial effects in the absence of light.[2,3]

Discussion

Non-viable bacteria were detected using a red fluorescent dye [DEAD—propidium iodide (PI)] which is membrane impermeant and as such is excluded from entering intact healthy cells. Viable bacteria were detected using a complementary green fluorescent dye (LIVE—SYTO9™) which is membrane permeable and stains all cells. When these dyes intercalate DNA, their fluorescence increases significantly; therefore, unbound dyes in the *milieu extérieur* do not interfere with the detection of the stains within bacterial cells. If both dyes are present in a cell (i.e. a bacterium with a damaged membrane), the DEAD stain displaces the LIVE stain from the nucleic acid due to its much higher affinity to intercalate DNA, and such a cell will fluoresce red (DEAD). The red colour channel was substituted for blue in

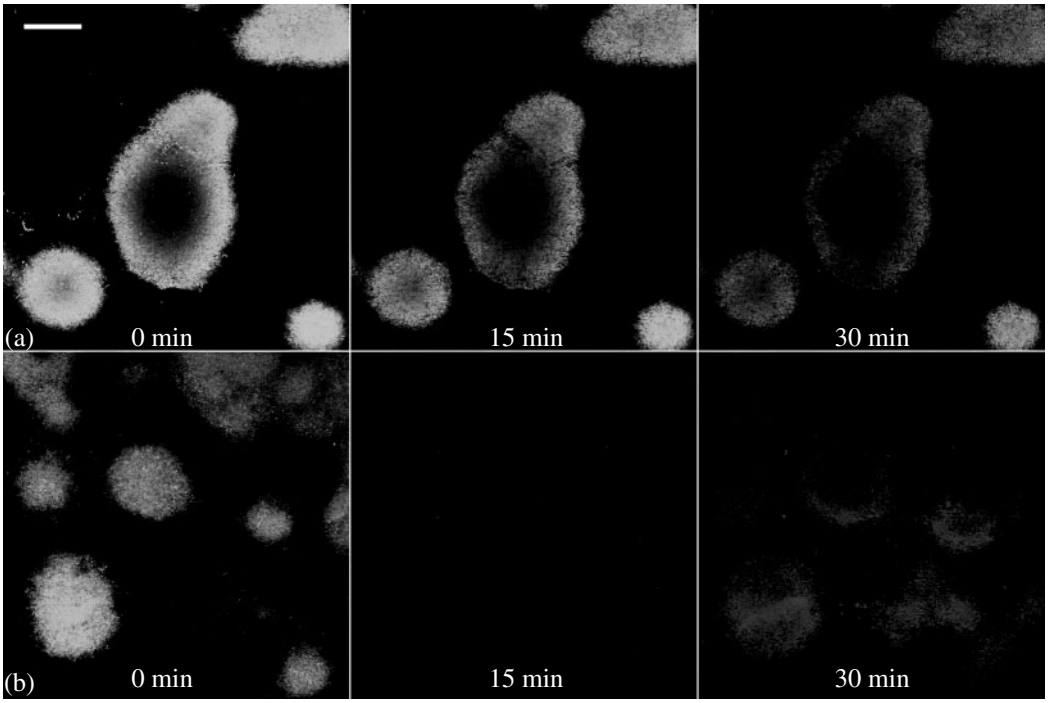

Figure 2. Optical sections through biofilms of *S. pyogenes* after repeated scanning/exposure using a confocal laser microscope (bar = 50 μm). Green = LIVE [all panels in (a) and the 0 min panel in (b)]; blue = DEAD (the normally red DEAD stain was substituted for blue for clarity) [30 min panel in (b)]. (a) PBS control. (b) SnCe6 at 50 mg/L. Primary laser (488 nm) energy dose, 1.05 J; energy density, 1.17 kJ/cm^2. A colour version of this figure is available as Supplementary data at *JAC* Online (http://jac.oxfordjournals.org/).

Figure 2 in order to better differentiate between viable and non-viable cells.

During the exposure of *S. pyogenes* biofilms in PBS to laser light, no net increase in the DEAD stain was detected. When SnCe6 was present at 50 mg/L, an increase in DEAD stain fluorescence was detected over successive exposure/recovery cycles. The increase in DEAD fluorescence was most significant in the later stages of the experiments, indicative of the cumulative damage that had occurred. Cell death was probably caused by the generation of $^1O_2$ by SnCe6 on exposure to laser light.[9]

The 488 nm Ar laser was nominated as the primary illumination source since it yielded a higher energy dose than the 543 nm HeNe laser, and as such it was also easier to make the necessary adjustments to its detector settings. The maximum absorption (0.941 units) of SnCe6 occurs at 633.5 nm, although it does adsorb light photons and therefore produces $^1O_2$ over a range of wavelengths, including 488 nm (0.06 units) and 543 nm (0.09 units) [Robert Cundell (Frontier Scientific), personal communication, June 2005]. Other confocal systems which incorporate a tunable laser would allow SnCe6, or indeed other photosensitizers, to be illuminated at their maximum absorption wavelengths. This would maximize the production of $^1O_2$, in a process that would be separate for the emissions required for the LIVE and DEAD stains. Although the absorption maximum of SnCe6 falls within the emission spectrum of PI, which peaks at around 615–630 nm, there is sufficient DEAD fluorescence emission beyond that of SnCe6 (>650 nm) for detection without significant shielding by the photosensitizer. The transfer of energy from PI emissions to SnCe6 by fluorescence resonance energy transfer is not likely to be significant when compared with the emissions caused by the laser light, an effect which will be minimized by the fact that PI is present at only 10 μM in the free solution and will be localized in the DNA, while SnCe6 will be dispersed throughout the viewing solution.

Lethal photosensitization was induced in biofilms of *S. pyogenes* when treated with a solution of SnCe6 (50 mg/L) and exposed to the 488 nm Argon and 543 nm HeNe laser emissions of a confocal microscope. These same scanning lasers simultaneously revealed the vital status of the cells by the incorporation of fluorescent indicators of membrane integrity. This coincident process allows the lethal photosensitization of biofilms to be evaluated in real-time and could be extrapolated to evaluate other existing and novel photosensitizers.

Acknowledgements

This work was funded by the Oral and Dental Research Trust: Proctor and Gamble Awards.

Transparency declarations

None to declare.

Supplementary data

A colour version of Figure 2 is available as Supplementary data at *JAC* Online (http://jac.oxfordjournals.org/).

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