The inability of a bacteriophage to infect *Staphylococcus aureus* does not prevent it from specifically delivering a photosensitiser to the bacterium enabling its lethal photosensitisation

Running Title: PDT Phage

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Abstract

Objectives: It has been demonstrated that the efficiency of lethal photosensitisation can be improved by covalently binding photosensitising agents to bacteriophage. In this study we have investigated whether a bacteriophage requires the capacity to infect the bacterium to enhance lethal photosensitisation when linked to a photosensitizer.

Methods: Tin (IV) chlorin e6 (SnCe6) was conjugated to bacteriophage Φ11, a transducing phage which can infect *Staphylococcus aureus* NCTC 8235-4, but not EMRSA 16. The conjugate and appropriate controls, were incubated with these bacteria and either exposed to laser light at 632.8 nm or kept in the dark.

Results: The SnCe6 / Φ11 conjugate achieved a statistically significant reduction in the number of viable bacteria of both 8325-4 and EMRSA 16 strains by 2.31 $\log_{10}$ and 2.63 $\log_{10}$ respectively. The conjugate could not however instigate lethal photosensitisation in *E. coli*. None of the other combinations of controls; such as an equivalent concentration of SnCe6 only, an equivalent titre of bacteriophage only or experiments conducted without laser light; yielded significant reductions in the number of viable bacteria recovered.

Conclusions: The inability of a bacteriophage to infect *S. aureus* does not prevent it from specifically delivering a photosensitiser to a bacterium enabling its lethal photosensitisation.

Introduction

Light-activated antimicrobial agents (photosensitisers) are an appealing alternative to conventional antibiotics for the treatment of localised bacterial infections. Lethal photosensitisation (LP) has been demonstrated to be effective at killing a range of bacteria including opportunistic pathogens, commensal cutaneous species,[1] periodontal pathogens[2] and epidemic methicillin-resistant *Staphylococcus aureus* (EMRSA).[3] LP has two main advantages over conventional antimicrobial chemotherapy. Firstly, the bactericidal effect is limited to the area that is treated with both the photosensitiser and light, preventing disturbance of the wider commensal microbial community.[4] Secondly, the non-specific mode of action of liberated singlet oxygen ($^1O_2$) against bacteria is unlikely to induce the development of protective mechanisms and the subsequent proliferation of these genes through the wider microbial community.

The inherent reactivity of $^1O_2$ limits its ability to diffuse through an aqueous environment. The lifetime of $^1O_2$ in pure water is ~4 μs, which results in a theoretical diffusion distance of 125 nm, if one assumes that the moiety does not interact with a biological molecule.[5] This short range action (on the scale of biological systems) may possibly limit the effectiveness of LP. We have previously developed methodologies to facilitate the close association of photosensitiser and bacteria using targeting systems based upon the covalent conjugation of the photosensitiser tin (IV) chlorin e6 (SnCe6) onto immunoglobulin G (IgG).[3, 6] More recently, we have found that covalently linking SnCe6 to *S. aureus* bacteriophage 75, commonly used for typing, targets lethal photosensitisation to a range of strains of *S. aureus* including MRSA.[7] In the study reported herein, we examined whether another

unrelated *S. aureus* bacteriophage, phage Φ11 a generalised transducing phage, could replace phage 75 in targeting lethal photosensitisation to *S. aureus* strains.

Materials and Methods

Bacteria and Bacteriophage

The two strains of *S. aureus* used in these experiments were EMRSA-16 (NCTC 13143), one of the dominant nosocomial MRSA isolates in UK hospitals and 8325-4, a prophage-free derivative of NCTC8325 which is methicillin-sensitive. These strains were grown on Columbia agar (Oxoid Ltd., Basingstoke, United Kingdom) supplemented with 5% (vol/vol) defibrinated horse blood (CBA). *E. coli* 10418 was also incorporated as a Gram-negative control. In preparation for the lethal photosensitisation experiments, a colony was inoculated into 20 mL of nutrient broth no. 2 (NB2) containing 10 mM $CaCl_2$ and grown aerobically for 16 hours at 37ºC in a shaking incubator. The cultures were then washed by centrifugation and re-suspension in PBS containing 10 mM $CaCl_2$ and adjusted to a final optical density of 0.05 at 600 nm (OD_{600}), these cell suspensions contained approximately 1 x 10^7 cfu/mL.

Bacteriophage Φ11 is a generalised transducing phage present in *S. aureus* NCTC 8235 as a prophage.[8] This bacteriophage can infect the NCTC8325 derivative 8325-4, but not EMRSA16. Phage Φ11 was propagated in *S. aureus* 8325-4 using the phage overlay method and SnCe6 was covalently conjugated to the bacteriophage using methods described previously.[7] The concentration of SnCe6 bound to the phage was determined by spectral analysis against a calibration curve generated from known concentrations of SnCe6. In different experiments, between 7.5 x 10^6

and 4.7 x 10^7 cfu of bacteriophage were used in conjugation reactions and the amount of SnCe6 bound varied between 3.5 and 7 µg/mL.

Lethal Photosensitisation Experiments

Fifty microlitres of the Φ11-SnCe6 conjugate was added to 50 µL of bacterial suspension in a sterile 96-well plate. The controls consisted of: SnCe6 alone (at the same concentration as the conjugate), Φ11 alone (at the same titre as the conjugate) and PBS control. All of the mixtures were incubated in the dark, with stirring, for 30 minutes prior to exposure to laser light. The relevant samples were then sequentially exposed to laser light (632.8 nm) from a helium / neon (HeNe) gas laser with a measured power output of 29.2 mW (Spectra-Physics, Darmstadt-Kranichstein, Germany) for a period of 5 minutes; the mixtures were magnetically stirred throughout the course of an experiment. Additional 'dark controls' were also conducted for these four variables without laser light. The number of viable bacteria remaining in the samples was determined immediately following exposure to the laser light by serial dilution and enumeration of colony forming units on CBA. Each experimental variable was repeated as a duplicate.

The duplicate experiments with both strains of *S. aureus* were conducted a total of four times (n=8), whilst those for *E. coli* were repeated twice (n=4). The null hypothesis was that there was no difference between the $\log_{10}$ counts of the number of colony forming units using various different experimental parameters, this was analysed by student's t-test to yield p-values.

Results

When compared to the control, which was not exposed to laser light nor to photosensitiser, the Φ11-SnCe6 conjugate in the presence of laser light yielded a 2.31 $\log_{10}$ reduction (p=<0.05) in the number of viable bacteria recovered from the culture of *S. aureus* 8325-4 and a 2.63 $\log_{10}$ reduction (p=<0.05) for the culture of EMRSA16. In the presence of laser light, the Φ11-SnCe6 conjugate did not result in significant killing of *E. coli* 10418. None of the other combinations of controls (i.e. SnCe6 only, phage only and 'dark controls') produced significant bacterial kills (figure 1).

Discussion

We have previously shown that bacteriophage 75, a serotype F staphylococcal phage, could be used to target lethal photosensitisation to a range of *S. aureus* strains including strains it could not infect. The capacity of bacteriophage 75 to target LP to a range of *S. aureus* strains was surprising since this phage has a restricted host range. The question we asked in the current study was whether other staphylophage could target LP to a range of *S. aureus* strains, once conjugated to a photosensitiser, or if this was a specific trait of phage 75. We did this by investigating the capacity of bacteriophage Φ11, a prototypic group B-transducing phage,[9] to target lethal photosensitisation to *S. aureus*.

When SnCe6 was conjugated to *S. aureus* bacteriophage Φ11, *S. aureus* strains 8325-4 and EMRSA16 in the presence of laser light there was an increase in the killing of these bacteria by 2.39 $\log_{10}$ and 2.35 $\log_{10}$ respectively, when compared to the equivalent concentration of SnCe6 alone (i.e. free SnCe6 that was not

conjugated to the bacteriophage). Since it is known that staphylophage have the capacity to bind to all strains of *S. aureus*[10] and bacteriophage Φ11 is not capable of infecting strain EMRSA16, the kill achieved by the Φ11-SnCe6 conjugate suggests that the photosensitiser-bacteriophage conjugate only needs to bind to the bacterial cell to induce killing in the presence of laser light. The selectivity of the photosensitier-bacteriophage conjugate in targeting lethal photosensitisation to *S. aureus* was demonstrated by the inability to cause significant killing of *E. coli* in the presence of laser light.

Our results demonstrate that it is possible to use different serotypes of staphylophage as vehicles to deliver photosensitiser payloads to the surface of *S. aureus* thus enabling selective lethal photosensitization of this bacterium in the presence of laser light. Such designer composites would not only possess all of the advantages that photodynamic therapy has over conventional antibiotic therapy, as described in the introduction, but they would also ensure there was minimal collateral damage to the host and its indigenous microflora.

Transparency Declarations

None

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Figure Legend

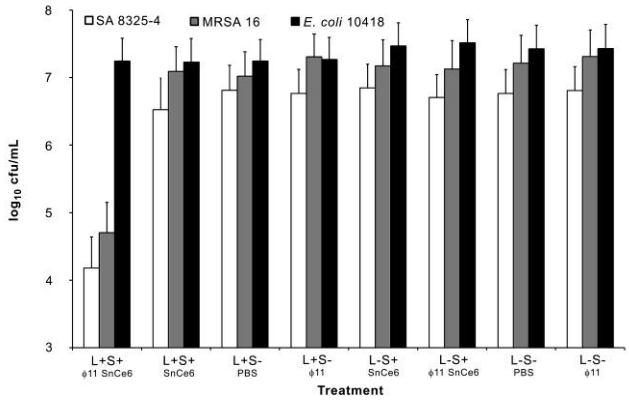

Figure 1. The number of viable bacteria recovered following exposure of SnCe6-bacteriophage 11 conjugate to laser light (leftmost columns) compared to controls. The designations L+ / L- and S+ / S- refer to the presence or absence of light and / or photosensitiser respectively.

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