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Brief reports

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**Killing of methicillin-resistant *Staphylococcus aureus in vitro* using aluminium disulphonated phthalocyanine, a light-activated antimicrobial agent**

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The aim of this study was to determine whether 16 epidemic methicillin-resistant strains of *Staphylococcus aureus* (EMRSA) could be killed by the light-activated antimicrobial agent aluminium disulphonated phthalocyanine (AIPcS<sub>2</sub>). EMRSA suspensions were irradiated with light from a laser diode in the presence of AIPcS<sub>2</sub> and survivors enumerated. All strains were susceptible to killing, the bactericidal effect being dependent on the AIPcS<sub>2</sub> concentration and the light dose. AIPcS<sub>2</sub> rendered the bacteria light-sensitive almost immediately and killing was unaffected by the growth phase of the organism. Scavengers of singlet oxygen and free radicals protected the bacteria from killing. These results imply that light-activated antimicrobial agents may be useful in eliminating EMRSA from wounds or carriage sites.

**Introduction**

Methicillin-resistant *Staphylococcus aureus* (MRSA) is a persistent and increasing cause of nosocomially acquired infections in many hospitals. The management of patients with infections due to MRSA (or carriers of the organism) usually involves the topical application of antimicrobial agents to eradicate carriage of the organism.<sup>1</sup> Unfortunately, there is now evidence of the development of resistance to many of these agents.<sup>2</sup> There is, therefore, a need to develop alternative means of eliminating *S. aureus* from infected wounds and carriage sites. One possible approach is to use light-activated antimicrobial agents to achieve lethal photosensitization of the organism; we have used these agents to kill *S. aureus in vitro*.<sup>3</sup> The purpose of this study was to determine whether the light-activated antimicrobial agent, aluminium disulphonated phthalocyanine (AIPcS<sub>2</sub>), could be used to kill 16 phage-typed epidemic strains of MRSA (EMRSA) when irradiated with low doses of red light.

**Materials and methods**

*Bacterial strains*

The organisms used in this study were the 16 phage-typed EMRSA strains 1–16 supplied by the National Collection of Type Cultures, Colindale, London, UK. They were grown aerobically in nutrient broth at 37°C for 16 h, harvested and resuspended in an equal volume of 0.85% (w/v) saline.

*Laser and photosensitizer*

A 9.25 mW indium/gallium/aluminium/phosphorous (In/Ga/Al/P) laser diode (LaserMax, New York, USA) emitting light with a wavelength of 673 nm was used. The photosensitizer was AIPcS<sub>2</sub> (absorption maximum 675 nm), kindly provided by Professor D. Phillips (Imperial College of Science, Technology and Medicine, London, UK).

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*Effect of photosensitizer concentration on lethal photosensitization*

Aliquots (100  $\mu$ L) of a saline suspension of strain EMRSA-12 (containing approximately  $5 \times 10^8$  cfu) were transferred to wells of a microtitre plate and an equal volume of AlPcS<sub>2</sub> in saline (0.85% w/v) was added to each well to give final concentrations ranging from 3.125 to 12.5 mg/L. The plate was placed on a magnetic stirrer and left for 60 s in the dark. Duplicate wells were then exposed to laser light for 120 s ( $I^+ s^+$ ), giving a light dose of 1.1 J (energy density = 41.7 J/cm<sup>2</sup>). Two control wells were not exposed to laser light to determine the effect on bacterial viability of the photosensitizer alone ( $I^- s^+$ ). Four additional wells containing the bacterial suspension plus saline instead of sensitizer solution were prepared, two of these were exposed to laser light for 120 s to determine the effect of laser light alone on bacterial viability ( $I^+ s^-$ ) while the remaining two were kept in the dark to determine the initial concentration of bacteria in the suspensions ( $I^- s^-$ ). After irradiation of appropriate wells, survivors were enumerated by viable counting on nutrient agar.

*Effect of pre-irradiation time*

Using 6.25 mg/L AlPcS<sub>2</sub> and a light dose of 1.1 J, the effect of varying the pre-irradiation time (PIT) between 0 and 300 s on the killing of EMRSA-12 was determined as described above.

*Effect of laser light dose*

The effect of laser light dose (varied by exposure for different periods of time) on the killing of EMRSA-12 was investigated using the above protocol with a PIT of 60 s and 6.25 mg/L AlPcS<sub>2</sub>.

*Susceptibility of other strains of EMRSA*

Using a PIT of 60 s, a light dose of 1.1 J and 6.25 mg/L AlPcS<sub>2</sub>, the susceptibility of the remaining 15 EMRSA strains was determined as described above. The operating parameters chosen were those unlikely to achieve 100% kills so that any differences in the susceptibility of the strains would be apparent.

*Susceptibility of irradiation survivors*

In some experiments, small numbers of colonies were seen on the plates derived from the  $I^+ s^+$  wells. In order to ascertain whether these bacteria constituted a photolysis-resistant subpopulation, colonies were resuspended in saline and their susceptibility to lethal photosensitization was determined by exposure to 1.65 J of laser light in the presence of AlPcS<sub>2</sub> (6.25 mg/L) using a PIT of 60 s.

*Effect of free-radical and singlet oxygen scavengers*

EMRSA-12 was suspended in saline only (controls), or varying concentrations of sodium azide (0.0005–0.5 M) or 1,4-diazabicyclo-[2,2,2]octane (DABCO; 0.005–0.1 M) and incubated at 4°C in the dark for 3 h. Duplicate wells were prepared for exposure to laser and sensitizer ( $I^+ s^+$ ) in the presence of saline (controls) or solutions of the scavengers. In order to detect any bactericidal activity of the scavengers themselves, similar duplicate wells were also prepared and were not exposed to laser light or to the AlPcS<sub>2</sub>. The procedure was then as described above but using a light dose of 1.65 J.

*Lethal photosensitization of EMRSA-12 in different growth phases*

Lag, logarithmic and stationary phase cultures of EMRSA-12 were identified on the basis of growth curves constructed from measurements of the optical density at 540 nm. Suspensions of cells from the three growth phases were prepared and experiments carried out using the following parameters: PIT = 60 s, 1.65 J of light, 6.25 mg/L of AlPcS<sub>2</sub>.

*Statistical analysis*

Student's *t*-test was used to compare the numbers of survivors in treated wells with the number of survivors in the control wells.  $P < 0.05$  was considered statistically significant.

**Results and discussion**

Killing of EMRSA-12 was found to be dependent both on the AlPcS<sub>2</sub> concentration and the light dose employed. Reductions in the viable count of a suspension containing  $4.53 \times 10^8$  cfu amounting to 4.52 log<sub>10</sub>, 5.22 log<sub>10</sub> and 3.23 log<sub>10</sub> were obtained in the presence of 12.5, 6.25 and 3.125 mg/L of AlPcS<sub>2</sub> respectively. Irradiation with 0.56 J of laser light resulted in a 3.36 log<sub>10</sub> reduction in the viable count while 100% kills (an 8.5 log<sub>10</sub> reduction) were obtained using 3.89 J. Altering the time (PIT) between addition of AlPcS<sub>2</sub> and exposure to laser light had little effect on the kills attained. With PITs of 0 s, 60 s and 300 s the log<sub>10</sub> reductions were 5.21, 5.84 and 4.43 respectively. These findings are encouraging from the clinical point of view as they imply that successful elimination of MRSA could be achieved using low doses of the drug and short (120 s) exposure times.

Irradiation of EMRSA strains 1–16 for 120 s (energy dose = 1.1 J) in the presence of 6.25 mg/L of AlPcS<sub>2</sub> resulted in substantial kills in all cases (Table). The mean reduction in the viable count was 5.48 log<sub>10</sub> but kills varied from a 2.67 log<sub>10</sub> reduction (EMRSA-9) to an 8.79 log<sub>10</sub> reduction (EMRSA-15). Interestingly, small but significant reductions in the viable counts of EMRSA-3, -4 and -16

**Table.** Reduction in viable counts of suspensions of the 16 EMRSA strains exposed to 1.1 J of laser light in the presence of AlPcS<sub>2</sub> at 6.25 mg/L

EMRSA strain no.	Reduction in viable count (log <sub>10</sub> )		
	I <sup>+</sup> s <sup>-</sup>	I <sup>-</sup> s <sup>+</sup>	I <sup>+</sup> s <sup>+</sup>
1	0	0	6.87
2	0.06	0	5.24
3	1.04	0.09	4.27
4	1.04	0	5.95
5	0	0.01	5.46
6	0.08	0	7.55
7	0	0.28	4.91
8	0.28	0	4.54
9	0	0	2.67
10	0.15	0.01	5.52
11	0.04	0	5.35
12	0.07	0	4.39
13	0	0.01	5.10
14	0	0.17	5.18
15	0	0	8.79
16	0.37	0.08	5.83
Mean	0.19	0.04	5.48

I<sup>+</sup> s<sup>+</sup> = suspensions exposed to laser light in the presence of AlPcS<sub>2</sub>;

I<sup>+</sup> s<sup>-</sup> = suspensions exposed to laser light in the absence of AlPcS<sub>2</sub>;

I<sup>-</sup> s<sup>+</sup> = unirradiated suspensions in the presence of AlPcS<sub>2</sub>.

were found when these were irradiated in the absence of AlPcS<sub>2</sub>. The only strain demonstrating any susceptibility to the AlPcS<sub>2</sub> in the absence of laser light was EMRSA-7, for which there was a 0.28 log<sub>10</sub> reduction.

In several of the experiments there were a few survivors. These might have represented a subpopulation resistant to photolysis. When these organisms were subjected to lethal photosensitization, similar kills to those achieved with the original suspensions were found. Hence, using an energy dose of 1.65 J and 6.25 mg/L AlPcS<sub>2</sub>, reductions in the viable count of 3.31 log<sub>10</sub> and 4.15 log<sub>10</sub> were achieved using survivors from different experiments. It is likely, therefore, that these survivors were not a resistant subpopulation but were simply organisms which had not been sensitized or had not received a light dose large enough to generate sufficient free radicals or singlet oxygen to ensure killing.

The hypothesized mechanism of lethal photosensitization is that laser light is absorbed by the sensitizer molecule, causing it to be raised from its stable ground state to a higher-energy triplet state. Interaction with neighbouring molecules then produces cytotoxic free radicals and/or singlet oxygen. Addition of sodium azide, a scavenger of free-radicals and singlet oxygen, reduced the kills in suspensions exposed to laser light and AlPcS<sub>2</sub>. In the absence of sodium azide there was a 5.28 log<sub>10</sub> reduction in the viable count but this kill was reduced in a dose-related manner with increasing concentrations of the scavenger.

The sodium azide itself did not affect the viable count of the EMRSA-12. Similar results were seen with DABCO, implying that killing was mediated by singlet oxygen and/or free radicals.

In the clinical setting, bacteria would be present in all three stages of growth and it is well-established that bacteria in the stationary phase of growth are less susceptible to many antimicrobial agents. The results of this study demonstrated that EMRSA-12 displayed similar susceptibilities to lethal photosensitization in all three phases of growth. Using a light energy dose of 1.65 J and an AlPcS<sub>2</sub> concentration of 6.25 mg/L, log<sub>10</sub> reductions of 5.30, 4.57 and 4.85 were achieved in suspensions of bacteria in the lag, log and stationary phases of growth respectively.

While the results of this study demonstrate that killing of *S. aureus* can be achieved using AlPcS<sub>2</sub> *in vitro*, the susceptibility of host cells to lethal photosensitization would need to be investigated before the technique could be evaluated in the clinical setting. However, there is evidence to suggest that this may not be a problem. It has been shown that, following systemic administration of AlPcS<sub>2</sub>, irradiation of rabbit gingiva with light from a copper vapour-pumped dye laser resulted in some ulceration which healed in 2 weeks.<sup>4</sup> However, the energy dose (20 J) and density (64 kJ/cm<sup>2</sup>) were approximately 18-fold and 1500-fold greater respectively than those used to obtain a bactericidal effect in the present study. Nevertheless, should it be demonstrated that injury to mammalian cells occurs at the energy doses and photosensitizer concentrations required to kill *S. aureus* then an alternative strategy would be required. Linking the photosensitizer to an anti-staphylococcal antibody could be a useful means of ensuring that it is taken up only by the bacterium, thus avoiding photosensitization of host cells. Specific targeting of *Pseudomonas aeruginosa* alone and in the presence of *S. aureus* using this approach has already been reported.<sup>5</sup>

In summary, the results of this study have demonstrated that low concentrations of AlPcS<sub>2</sub> rendered all 16 EMRSA strains susceptible to killing by low doses of laser light and that there were no marked differences in susceptibility among the strains. The extent of killing was dependent both on the light energy dose delivered and the photosensitizer concentration employed while it was unaffected by the pre-irradiation time or the growth phase of the organism. If similar activity can be demonstrated *in vivo*, this method may offer an alternative to traditional antimicrobial agents for the elimination of the organism from wounds, burns and carriage sites. The photosensitizer could be applied directly to the wound or carriage site (nares, nasopharynx, etc.) and then the site irradiated with light from the low-power laser. The advantages of lethal photosensitization would be a rapid bactericidal effect which is highly localized and would not disrupt the microflora at other sites; while the bacteria would be unlikely to develop resistance as killing is mediated by singlet oxygen and free radicals.

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