Consequences of daptomycin-mediated membrane damage in *Staphylococcus aureus*

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**Objectives**: The proposed lethal action of daptomycin on *Staphylococcus aureus* results from the loss of K\(^+\) and membrane depolarization. However, whether these events alone cause cell death has been questioned. We sought to determine whether other consequences of daptomycin-mediated membrane damage may contribute to cell death.

**Methods**: Previously established assays were used to evaluate the membrane damaging activity of daptomycin at a single time-point of 10 min. More detailed time-course experiments were also performed to determine the kinetics of membrane depolarization and leakage of K\(^+\), Mg\(^{2+}\) and ATP. The kinetics of inhibition of macromolecular synthesis following exposure to daptomycin were also determined by assaying the incorporation of radioactive precursors into macromolecules.

**Results**: Daptomycin exhibited no membrane damaging activity in single time-point assays following exposure to the antibiotic for 10 min. Kinetic analysis confirmed these results as leakage of intracellular components did not occur until 20–30 min, membrane depolarization was gradual and cells remained biosynthetically active for at least 30 min after exposure to daptomycin. Viability declined rapidly after exposure to daptomycin and appeared to precede other detectable changes.

**Conclusions**: These data show that daptomycin-induced loss of Mg\(^{2+}\) and ATP occurs in conjunction with the previously reported leakage of K\(^+\) and membrane depolarization. We propose that the lethal activity of daptomycin is not simply due to loss of K\(^+\) and probably involves more general damage to the membrane.

Keywords: membrane potential, macromolecular synthesis, *S. aureus*

**Introduction**

Daptomycin is a cyclic lipopeptide antibiotic with bactericidal activity used in the treatment of serious Gram-positive infections, particularly those caused by *Staphylococcus aureus*. Its mode of action (MOA) is controversial. Suggested mechanisms include inhibition of lipoteichoic acid biosynthesis, interaction with RNA polymerase, disruption of cell division and inhibition of peptidoglycan biosynthesis. Although these mechanisms may contribute to the MOA of daptomycin, the lethal activity of the antibiotic appears to result primarily from the disruption of the cytoplasmic membrane, and a direct correlation between daptomycin-induced membrane perturbation and cell death has been claimed by Silverman *et al.*

The Silverman model for the MOA of daptomycin is a multi-step model, and the initial stage involves binding and insertion of daptomycin into the cytoplasmic membrane in the presence of Ca\(^{2+}\). Ca\(^{2+}\) probably acts as a cross-bridge between the negatively charged daptomycin molecules and the membrane, and promotes deeper insertion of daptomycin into the bilayer. Daptomycin molecules may then oligomerize within the membrane to form a channel through which K\(^+\) is lost, leading to membrane depolarization and cell death. This model has been further supported by Straus and Hancock, who revised it based on structural studies and suggested that daptomycin in the presence of Ca\(^{2+}\) may aggregate to form micelles prior to membrane insertion.

Despite data supporting the Silverman model, its accuracy has been questioned, and in particular, whether membrane depolarization alone is sufficient to cause cell death. The Silverman model is based on an apparent correlation between cell death (measured by viable count), K\(^+\) leakage and membrane depolarization. However, the experiments by which these
events were detected were not conducted under comparable conditions, and other measurements of membrane integrity were not considered. In addition, a recent study has shown that contrary to the work of Silverman et al., prolonged exposure to lethal concentrations of daptomycin only resulted in partial membrane depolarization of S. aureus. 10

Previous work from this laboratory has established a set of relatively simple methods to detect agents causing membrane damage in S. aureus involving fixed time-point assays to detect leakage of intracellular components and the BacLight fluorescent dye system for measuring membrane integrity.11 However, daptomycin has not been examined in these fixed time-point assays. In view of the inconsistencies regarding the MOA of daptomycin discussed above, we initially investigated the effects of daptomycin on S. aureus using single time-point assays for membrane damage. The results of these experiments led us to re-examine the timing of K⁺ leakage and other intracellular components, membrane depolarization, loss of viability and inhibition of biosynthetic activity in an attempt to determine the sequence of events that occur following exposure of S. aureus to daptomycin.

Materials and methods

Bacterial strains, growth media, antibiotics and chemicals
S. aureus 8325-412 was used for all experiments and routinely grown in Mueller–Hinton broth (MHB) (Oxoid, Basingstoke, UK) supplemented with 50 mg/L Ca²⁺. Plasmid pAJ22, encoding β-galactosidase,11 was transformed into S. aureus 8325-4, and the resultant strain was used in the β-galactosidase leakage assay. During macromolecular synthesis assays, MHB was found to interfere with the incorporation of [5,6-3H]uridine into RNA and so MHB was replaced with Luria–Bertani (LB) broth (Oxoid), supplemented with 50 mg/L Ca²⁺, for all macromolecular synthesis assays. Antibiotics and chemicals were from Sigma-Aldrich (Poole, UK) with the exception of daptomycin (Chiron, Oxford, UK), DiSC₃(5) and the ATP Determination Kit (both from Invitrogen Life Technologies, Paisley, UK). The following radiolabelled chemicals were from GE Healthcare (Little Chalfont, Buckinghamshire, UK): [methyl-3H]thymidine (70–95 Ci/mmol), [5,6-3H]uridine (31–56 Ci/mmol), L-[G-3H]glutamine (20–50 Ci/mmol) and [1-14C]glycine (50–62 mCi/mmol).

MIC determinations

MIC values were determined by 2-fold dilutions of antibacterial agents in MHB according to the broth microdilution guidelines set out by the CLSI (formerly the NCCLS).13

Fixed time-point measurements of membrane damage

Measurements of membrane integrity using BacLight and release of ATP and β-galactosidase were made following a 10 min exposure to daptomycin (in the presence of 50 mg/L Ca²⁺) or comparator antibiotics at 4 × MIC as described previously.11

Measurements of viability, leakage of intracellular components and membrane potential over a time-course

The effects of daptomycin on membrane integrity and the recovery of viable cells were examined by performing assays over a time-course on bacteria resuspended in 5 mM HEPES buffer (pH 7.2), supplemented with 5 mM glucose and 50 mg/L Ca²⁺. Viable count determinations were performed on Mueller–Hinton agar (Oxoid), as described previously.14

Leakage of K⁺ and Mg²⁺ from cells following antibiotic exposure was monitored by atomic absorption spectroscopy, as described previously.15 The concentration remaining in the cells at each time-point was expressed as a percentage of the total (determined by boiling an aliquot of the starting culture for 10 min).16 Total ion contents were ~2 and ~0.15 mg/L for K⁺ and Mg²⁺, respectively. ATP concentrations in both the supernatant and the cell pellet were measured as described previously,17 and intracellular ATP concentrations were expressed as a percentage of the total.

Macromolecular synthesis assays

The incorporation of radiolabelled precursors into DNA ([methyl-3H]thymidine), RNA ([5,6-3H]uridine), protein (L-[G-3H]glutamine) and peptidoglycan ([1-14C]glycine) was monitored for 2 h. Cultures were pulse-labelled with the precursor (1 μCi/mL for 3H-labelled compounds and 0.1 μCi/mL for [1-14C]glycine) for 10 min before the addition of antibiotics. At intervals, culture samples were mixed with ice-cold 10% trichloroacetic acid (TCA) and stored on ice for 30 min to precipitate macromolecules. Samples were processed and counted as described previously.18

Results and discussion

Evaluation of the membrane damaging effects of daptomycin in fixed time-point (10 min) assays

We have previously evaluated a range of methods to assess membrane perturbation and gross membrane damage in S. aureus.11 We applied three of the most sensitive and discriminatory assays to study the MOA of daptomycin (Table 1).

As expected, the non-lytic agent, tetracycline, gave a negative result in the BacLight assay and caused no leakage of ATP or β-galactosidase. Nisin, which is a potent pore-forming antibiotic,22 reduced membrane integrity to zero in the BacLight assay and caused extensive leakage of the relatively small molecule ATP (MW: 507) and some leakage of the large protein β-galactosidase (MW: 464 000). The K⁺ ionophore valinomycin and the electron transport uncoupler carbonyl cyanide m-chlorophenylhydrazone (CCCP) both gave strong positive results in the BacLight assay, although they did not cause a leakage of ATP or β-galactosidase. Overall, these results suggest that the BacLight assay can detect the dissipation of the membrane potential as well as the physical membrane damage, at least in the case of nisin.

Daptomycin had no effect on membrane integrity as measured by the BacLight assay. Within 10 min, daptomycin also did not cause leakage of either ATP or β-galactosidase (Table 1).
Leakage of intracellular components and membrane depolarization following exposure to daptomycin

Our initial findings suggested that the ability of daptomycin to dissipate the membrane potential was not as potent as reported previously. We also suspected that $K^+$ might not be the only intracellular component released following daptomycin treatment. Therefore, we quantified the leakage of $K^+$ and $Mg^{2+}$ from cells at time intervals following the addition of daptomycin and also determined the membrane potential of these cells.

Despite the lack of ATP leakage following daptomycin exposure in the single time-point assay, the leakage of ATP was monitored over the longer time-course. We also determined the viability of bacteria exposed to daptomycin. As previously proposed, viability was defined as the ability of cells to divide and form colonies on agar. All assays were performed in buffer under directly comparable conditions.

Figure 1 shows the relationships between viability, leakage of cytoplasmic components and membrane potential in *S. aureus*, following treatment with daptomycin and comparator antibiotics.

### Table 1. Membrane damage in *S. aureus* following exposure to daptomycin and comparator antibiotics at $4 \times$ MIC for 10 min

<table>
<thead>
<tr>
<th>Drug</th>
<th>MIC (mg/L)</th>
<th>BacLight reading (% membrane integrity)</th>
<th>relative ATP leakage</th>
<th>relative $\beta$-galactosidase release</th>
</tr>
</thead>
<tbody>
<tr>
<td>Drug-free control</td>
<td>—</td>
<td>100%</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Tetracycline</td>
<td>0.25</td>
<td>$98.93 \pm 4.52$</td>
<td>$0.92 \pm 0.12$</td>
<td>$0.93 \pm 0.09$</td>
</tr>
<tr>
<td>Nisin</td>
<td>2</td>
<td>$0.07 \pm 0.07$</td>
<td>$74.84 \pm 2.65$</td>
<td>$4.94 \pm 1.03$</td>
</tr>
<tr>
<td>Valinomycin</td>
<td>1</td>
<td>$38.58 \pm 2.30$</td>
<td>$0.88 \pm 0.10$</td>
<td>$0.92 \pm 0.07$</td>
</tr>
<tr>
<td>CCCP</td>
<td>1</td>
<td>$24.78 \pm 1.86$</td>
<td>$0.94 \pm 0.10$</td>
<td>$0.89 \pm 0.11$</td>
</tr>
<tr>
<td>Daptomycin (+Ca^{2+})</td>
<td>1</td>
<td>$108.37 \pm 19.80$</td>
<td>$0.81 \pm 0.08$</td>
<td>$1.30 \pm 0.58$</td>
</tr>
</tbody>
</table>

Determinations were performed in triplicate.
over a time-course. All agents were added at 4× MIC and the data shown are the mean of three replicates. As predicted from the single time-point membrane damage assays (Table 1), a rapid decrease in the viability induced by nisin was accompanied by leakage of cytoplasmic components (K⁺, Mg²⁺, ATP) and complete membrane depolarization (Figure 1a). Tetracycline, a bacteriostatic agent, had no effect on viability or membrane permeability (Figure 1b). Moxifloxacin, a bactericidal agent with an intracellular target, caused a reduction in viability but without any substantial membrane damaging effects (Figure 1c).

The profile of daptomycin (Figure 1d) is clearly distinct from those of the other antibiotics tested, in particular, nisin that is often used as a comparator to daptomycin.⁴,⁶,⁷,²⁴,²⁵ Daptomycin exhibited rapid bactericidal activity, causing a 99% reduction in

Figure 2. Effect of daptomycin on (a) cell viability, (b) DNA synthesis, (c) RNA synthesis, (d) protein synthesis and (e) peptidoglycan synthesis in S. aureus. Drug-free control, filled squares; 4 mg/L daptomycin, open circles; control inhibitors (b, 0.5 mg/L ciprofloxacin; c, 0.008 mg/L rifampicin; d, 0.25 mg/L tetracycline; e, 16 mg/L fosfomycin), filled triangles.
viability after 10 min. K$^+$ and Mg$^{2+}$ began to be lost after 10 min of exposure to daptomycin, followed by the release of ATP at 20 min and thereafter. Initially, daptomycin only induced relatively small changes in the membrane potential, and substantial membrane depolarization (>50%) was not evident until 60 min (Figure 1d). The slow rate of daptomycin-induced membrane depolarization reported here is in agreement with the results of Jones et al.10 The relatively slow onset of membrane damage induced by daptomycin explains why the 10 min single time-point assays (Table 1) failed to detect interference with membrane function. Overall, the time-course data indicate that K$^+$ is not the only intracellular component that is released following exposure to daptomycin, and that unlike the previously proposed model,7 daptomycin does not simply cause K$^+$ leakage and membrane depolarization.

Daptomycin does not cause immediate inhibition of biosynthetic activity

The viability data described earlier were generated following the plating of daptomycin-exposed cells onto agar. It is therefore a measurement of the subsequent ability of cells to divide and form colonies. Although the loss of viability following exposure to daptomycin appeared to occur very rapidly (Figure 1d), viability, as defined here, is actually a measure of the ability of daptomycin-treated cells to form colonies on agar 18–24 h after contact with the antibiotic. Therefore, the precise timing of death cannot be established by plating techniques. Other possible real-time measures of viability, such as membrane integrity26 and intracellular ATP content,27 could not be applied to daptomycin-treated cells due to the membrane damaging effects of the antibiotic. Therefore, the capacity of the cells to synthesize macromolecules was used as an indicator of the likely viability of the organism. These experiments could not be performed in buffer as they require cells to be metabolically active to incorporate the radiolabelled precursors. Therefore, it was important to determine that daptomycin retained potent bactericidal activity in LB broth under the conditions to be used for macromolecular synthesis assays. Daptomycin was rapidly bactericidal in both buffer and growth medium, although the rate of loss of viability was slower in the growth medium, with an apparent 94% reduction in viability within 30 min (Figure 2a).

Figure 2(b–e) shows the continued incorporation of labelled precursors into DNA, RNA, protein and peptidoglycan over the time-course by the drug-free control, and the rapid inhibition of each biosynthetic pathway by an appropriate positive control agent. In the presence of daptomycin, incorporation of all four precursors continued at similar rates as the drug-free controls for ~30 min. Daptomycin showed no preferential inhibition of any of the pathways.

Although the data from Figures 1(d) and 2(b–e) cannot be directly compared due to differences in the experimental conditions, ATP leakage and membrane potential were investigated with cells in the growth medium, and the timing and degree of leakage and membrane dissipation were very similar to those produced in buffer (data not shown). If the kinetics of cytoplasmic leakage are similar in the growth medium and buffer, then the inhibition of biosynthetic activity observed at ~30 min may result from the depletion of essential ions and/or ATP. If, as suggested previously,7 daptomycin causes specific leakage of K$^+$, preferential inhibition of protein synthesis might be expected as this process is particularly susceptible to K$^+$ depletion.28 However, as discussed earlier, all macromolecular synthetic processes were similarly affected in bacteria exposed to daptomycin (Figure 2).

When the data on cell survival, membrane damage and biosynthetic activity are considered together, it appears that the loss of viability induced by daptomycin precedes the leakage of cytoplasmic components, membrane depolarization and loss of biosynthetic activity, i.e. these events may not be responsible for the loss of viability. This is in contrast to the work of Silverman et al.,7 who reported that the loss of viability, K$^+$ leakage and membrane depolarization occurred simultaneously. In contrast to the earlier study,7 our experiments were performed under directly comparable conditions and may, therefore, provide a more accurate determination of events. Although our data suggest that the loss of viability precedes membrane damage, this may reflect the inability to follow loss of viability in real time such that cellular death, disruption of membrane integrity and inhibition of macromolecular synthesis are in fact simultaneous processes. Although the exact events that lead to daptomycin-induced loss of viability cannot be determined from the data currently available, it is not simply a consequence of K$^+$ leakage or membrane depolarization, but probably involves more generalized disruption of membrane permeability.

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