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Daptomycin in vitro activity against methicillin-resistant staphylococcus aureus is enhanced by D-cycloserine in a mechanism associated with a decrease in cell surface charge

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Daptomycin in vitro activity against methicillin-resistant \textit{Staphylococcus aureus} is enhanced by D-cycloserine in a mechanism associated with a decrease in cell surface charge

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**Running head:** Daptomycin activity enhanced by D-cycloserine.

**Keywords:** Methicillin-resistant \textit{Staphylococcus aureus}
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Abstract:

The killing activity of daptomycin against an isogenic pair of daptomycin susceptible and daptomycin non-susceptible (DNS) MRSA strains was enhanced by the addition of certain cell wall agents at 1xMIC concentrations. However, when high inocula of DNS were used, no significant killing was observed in our experiments. Cytochrome c binding assays revealed D-cycloserine as the only agent associated with a reduction in the cell surface charge of both strains, at the concentrations used.
Manuscript

The mechanisms of resistance to daptomycin in *Staphylococcus aureus* have been related to cell membrane alterations; cell wall thickening, modifications in membrane lipid composition, altered drug binding and changes in the surface charge (1).

Almost all synthesis of (the positively charged) D-alanine in *S. aureus* is controlled by alanine racemase (2,3). D-cycloserine (DCS) is a D-alanine analog used as a second-line treatment for mycobacterial infections. It prevents peptidoglycan synthesis in bacterial cell wall by a competitive inhibition of both alanine racemase and D-alanine ligase enzymes (4,5).

The aim of this study was to ascertain the effect of DCS, in comparison to other cell wall agents (CWA), on the activity of daptomycin against MRSA and to assess related variations in cell surface charge. For this purpose, an isogenic pair of MRSA strains from an episode of bacteremia and osteomyelitis treated with daptomycin was used: the daptomycin-susceptible progenitor, A8796 (DS) and the daptomycin-non susceptible derivative, A8799 (DNS), which harbored an *mprF* mutation (S337L) (6).

Daptomycin (Cubist Pharmaceuticals, Inc, Lexington, MA) and CWA were used in this study: vancomycin (VAN), ampicillin (AMP), oxacillin (OXA), cefazolin (CFZ), imipenem (IMI), fosfomycin (FOM) and D-cycloserine (DCS) (Sigma-Aldrich Corporation, St.Louis MO). For all experiments the purified powder of each antibiotic was resuspended following the CLSI recommendations (7).

**Determination of Minimum inhibitory concentrations (MICs) and studies of daptomycin susceptibility.** MICs of daptomycin, DCS and FOM were determined by broth macrodilution method according to standard recommendations (7). MICs of all other antibiotics were determined by the E-test (AB BioMerieux, Solna, Sweden),
according to the manufacturer’s instructions (Table 1). A see-saw effect (8,9) between the DAP MIC (increased) and the MICs of beta-lactams (decreased) was observed for the isogenic isolates (Table 1). Although this phenomenon is not completely understood, it might be related to an alteration of \textit{mecA} gene regulation in the DNS isolates (9).

\textbf{48-hour time-kill curves (TKC)} were performed following standard methodology (10). Standard inoculum (10^6 CFU/ml) and high inoculum (10^8 CFU/ml) of bacteria were used. In order to mimic clinically-relevant serum concentrations, 8 µg/mL (D8) of DAP were used in all experiments (11), either alone or in combination with CWA at prefixed concentrations of 1xMIC. Daptomycin alone and all the CWA showed no net killing activity at 48 hours against both strains. Against standard inoculum DS the addition of all CWA to DAP produced greater killing compared to D8 alone, but bactericidal activity was only maintained at 48h with the combinations OXA+D8 (Δ-3.93log), DCS+D8 (Δ-3.85log) and CFZ+D8 (Δ-3.17log). Bacterial regrowth at 48 hours was only prevented by the combinations DCS+D8 and OXA+D8 (Figure 1A). Against high inoculum DS bactericidal activity at 24h was only maintained at 48h with the combinations OXA+D8 (Δ-4.73log), DCS+D8 (Δ-5.85log) and IMI+D8 (Δ-4.64log) but regrowth was only prevented by the combination DCS+D8 (Figure 1B). Against standard inoculum DNS, the only combinations that prevented regrowth at 48 hours were OXA+D8 (Δ-3.80log), IMI+D8 (Δ-3.66log) and DCS+D8 (Δ-3.77log) (Figure 1C). As shown in figure 1D, against high inoculum DNS, synergy was not observed with any combination. In summary, at the concentrations used OXA, DCS and IMI had a comparable effect on daptomycin killing, there being synergistic or additive activity with D8 in three of the experiments (with the exception of DNS at initial high inoculum). However, DCS+D8 did not allow regrowth at 48 hours in any of those three experiments, and was the only
combination achieving net killing against the DNS strain at initial high inoculum (Δ-0.48log). As far as we know, this is the first time that DCS+daptomycin activity has been tested against MRSA. Synergism between daptomycin and beta-lactams or fosfomycin has been previously observed (12-15) and is further supported from our experiments.

**Evaluation of bacterial surface charge** (16). After overnight growth in trypticase soy broth (TSB), cultures were resuspended in fresh medium containing one CWA at 1xMIC. It was then resuspended in fresh medium containing the positively charged molecule cytochrome c (CyC) from bovine heart (Sigma). The optical density (A405) of the supernatant was measured (SPECTRAmax Plus 384, Molecular Devices Corporation, Sunnyvale, CA) and the total amount of cell-bound CyC was obtained using a standard curve. Experiments were repeated 4 times. Analysis of variance test with the post hoc Bonferroni correction were used to determine differences in the bound CyC means in the presence of various antibiotics. Differences were considered statistically significant when P values were ≤0.05. In the absence of antibiotic exposure, significant differences (p<0.05) were found between the amount of CyC bound to A8796 (1.39mg± 0.07) and A8799 (1.22mg±0.09) (Figures 2A and 2B). For both strains, the amount of CyC bound in the presence of DCS at 1xMIC was significantly higher compared to that bound in its absence (1.26-fold increase for A8796 and 1.68-fold increase for A8799). We hypothesize that the reduction in D-alanine synthesis achieved by DCS may be related to the reduction in the cell surface charge, signified by the increased CyC binding. As observed in figure 2B, for A8799 there were also significant differences regarding CyC binding in the presence of CFZ (1.21-fold increase) and FOM (1.09-fold increase), which might be strain-dependent. Also, since FOM inhibits the uridine diphosphate (UDP)-N-acetylglucosamine enolpyruval transferase, these results stress the concept that reduction
in the envelope charge is not specific to the mechanism of action of either DCS or the beta-lactams. They also suggest that mechanisms additional to surface charge changes are likely to be involved into synergism between CWA and daptomycin. Finally, our results highlight the importance of the bacterial inoculum for daptomycin efficacy and the difficulties to reach its targets in high bacterial density (17-19). This study has some limitations. Given the profound see-saw effect observed with the beta-lactams, their concentrations used against the DNS isolate were lower than those clinically achievable. The potential neurotoxicity and contraindication of DCS in severe renal impairment could severely limit its clinical use in treating severe MRSA infections. Nonetheless, this study does suggest a way of overcoming MRSA daptomycin resistance and opens the door to further research, based on reducing cell wall D-alanine composition, by inhibiting alanine racemase or other steps in cell wall alanylation.
References


Table 1: Daptomycin and cell wall agents’ minimum inhibitory concentrations (MIC) of a MRSA isogenic pair of strains

(*) MICs were determined two times. It was not detected significant increase in MIC at 48 hours with respect to that read at 24 hours, for any of the antibiotics tested.

<table>
<thead>
<tr>
<th></th>
<th>Daptomycin</th>
<th>Vancomycin</th>
<th>Ampicillin</th>
<th>Oxacillin</th>
<th>Cefazolin</th>
<th>Imipenem</th>
<th>Fosfomycin</th>
<th>D-cycloserine</th>
</tr>
</thead>
<tbody>
<tr>
<td>A8796</td>
<td>0.5</td>
<td>1</td>
<td>16</td>
<td>512</td>
<td>128</td>
<td>64</td>
<td>2</td>
<td>32</td>
</tr>
<tr>
<td>A8799</td>
<td>2</td>
<td>2</td>
<td>6</td>
<td>0.38</td>
<td>32</td>
<td>0.094</td>
<td>1</td>
<td>32</td>
</tr>
</tbody>
</table>

(*) MICs were determined two times. It was not detected significant increase in MIC at 48 hours with respect to that read at 24 hours, for any of the antibiotics tested.
Figure 1. 48-hours time-kill curve experiments with an isogenic pair of methicillin-resistant Staphylococcus aureus strain.
Figure 2: Cytochrome C (CYC) binding assays with an isogenic pair of methicillin-resistant Staphylococcus aureus strains, exposed to different cell wall agents at 1xMIC.