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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The killing activity of daptomycin against an isogenic pair of daptomycin-susceptible and daptomycin-nonsusceptible (DNS) methicillin-resistant *Staphylococcus aureus* (MRSA) strains was enhanced by the addition of certain cell wall agents at 1 × MIC. However, when high inocula of the DNS strain 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 for both strains at the concentrations used.

The mechanisms of resistance to daptomycin (DAP) in *Staphylococcus aureus* have been related to cell membrane alterations such as 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 the bacterial cell wall by competitive inhibition of both the 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 methicillin-resistant *S. aureus* (MRSA) and to assess related variations in cell surface charge. For this purpose, the following isogenic pair of MRSA strains from an episode of bacteremia and osteomyelitis treated with daptomycin was used: a daptomycin-susceptible (DS) progenitor, A8796, and a daptomycin-nonsusceptible (DNS) derivative, A8799, which harbored an *mprF* mutation (S337L) (6).

Daptomycin (Cubist Pharmaceuticals, Inc., Lexington, MA) and the following 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 standard methodology (10). A standard inoculum (10^6 CFU/ml) of DAP was used in all experiments (11), either alone or in combination with CWA at prefixed concentrations (1 × MIC). Daptomycin alone and all the CWA showed no net killing activity at 48 h against both strains. Against the standard inoculum of the DS strain, bactericidal activity at 48 h against both strains. Against the standard inoculum of the DS strain, bactericidal activity at 48 h was prevented only by the DCS-D8 and OXA-D8 combinations (Fig. 1A). Against the high inoculum of the DS strain, bactericidal activity at 24 h was maintained at 48 h only with the OXA-D8 (Δ = 3.93 log), DCS-D8 (Δ = 3.85 log), and CFZ-D8 (Δ = 3.17 log) combinations. Bacterial regrowth at 48 h was prevented only by the DCS-D8 and OXA-D8 combinations (Fig. 1A).

**TABLE 1** Daptomycin and cell wall agent MICs for an isogenic pair of MRSA strains

<table>
<thead>
<tr>
<th>Strain</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>

a MICs were determined two times. There was no significant increase in MIC at 48 h with respect to that read at 24 h for any of the antibiotics tested.
growth was prevented only by the DCS-D8 combination (Fig. 1B). Against the standard inoculum of the DNS strain, the only combinations that prevented regrowth at 48 h were OXA-D8 (∆-3.80 log), IMI-D8 (∆-3.66 log), and DCS-D8 (∆-3.77 log) (Fig. 1C). As shown in Fig. 1D, against the high inoculum of the DNS strain, synergy was not observed with any combination. In summary, at the concentrations used, OXA, DCS, and IMI had comparable effects on daptomycin killing, with synergistic or additive activity in three of the experiments (with the exception of the DNS strain at the initial high inoculum). However, DCS-D8 did not allow regrowth at 48 h in any of these three experiments and was the only combination achieving net killing against the DNS strain at the initial high inoculum (∆-0.48 log). 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 observed previously (12–15) and is further supported by 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 1×MIC. Each culture was then resuspended in fresh medium containing the positively charged molecule cytochrome c (CyC) from bovine heart (Sigma). The A<sub>405</sub> 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 with the post hoc Bonferroni correction was used to determine differences in the mean amounts of bound CyC 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 amounts of CyC bound to strains A8796 (1.59 ± 0.07 mg) and A8799 (1.22 ± 0.09 mg) (Fig. 2A and B). For both strains, the amount of CyC bound in the presence of DCS at 1×MIC was significantly higher than 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 cell surface charge signified by the increased CyC binding. As observed in Fig. 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 UDP-N-acetylgalactosamine 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 beta-lactams. They also suggest that mechanisms additional to surface charge changes are likely to be involved in synergism between CWA and daptomycin. Finally, our results highlight the impor-

FIG 1 Forty-eight-hour time-kill curve experiments with an isogenic pair of methicillin-resistant Staphylococcus aureus strains. A8796, daptomycin-susceptible strain (MIC, 0.5 μg/ml); A8799, daptomycin-nonsusceptible strain (MIC, 2 μg/ml). The initial standard inoculum was 6 log, and the initial high inoculum was 8 log, D8, DAP concentration of 8 μg/ml. All cell wall agents were used at 1×MIC.

FIG 2 Cytochrome C (CyC) binding assays with an isogenic pair of methicillin-resistant Staphylococcus aureus strains exposed to different cell wall agents at 1×MIC. Statistically significant differences with respect to the control mean, determined by post hoc ANOVA with the Bonferroni correction, are indicated.
tance of the bacterial inoculum for daptomycin efficacy and the difficulties in reaching its targets at a high bacterial density (17–19).

This study has some limitations. Given the profound seesaw effect observed with the beta-lactams, their concentrations used against the DNS isolate were lower than those that are clinically achievable. The potential neurotoxicity and contraindication of DCS for those with 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 the cell wall D-alanine composition by inhibiting alanine racemase or other steps in cell wall alanylation.

REFERENCES


