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

O. Gasch,a,b,c S. K. Pillai,a,b J. Dakos,a S. Miyakis,a,b,d R. C. Moellering, Jr.,a,b G. M. Eliopoulosa,b
Beth Israel Deaconess Medical Centera and Harvard Medical School,b Boston, Massachusetts, USA; Hospital Universitari de Bellvitge, Barcelona, Spain;c Graduate School of Medicine, University of Wollongong, New South Wales, Australia4

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 CLSI recommendations (7).

Determination of MICs and studies of daptomycin susceptibility. MICs of daptomycin, DCS, and FOM were determined by the broth macrodilution method according to standard recommendations (7). MICs of all other antibiotics were determined by use of Etest (AB bioMérieux, Solna, Sweden) according to the manufacturer’s instructions (Table 1). A seesaw 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 mecA gene regulation in DNS isolates (9).

Forty-eight-hour time-kill curves (TKC) were performed following standard methodology (10). A standard inoculum (10⁶ CFU/ml) and a high inoculum (10⁸ CFU/ml) of bacteria were used. In order to mimic clinically relevant serum concentrations, 8 µg/ml (D8) 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, the addition of all CWA to DAP produced greater killing than that with D8 alone, but bactericidal activity 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). Against the high inoculum of the DS strain, bactericidal activity at 24 h was maintained at 48 h only with the OXA-D8 (Δ = 4.73 log), DCS-D8 (Δ = 5.85 log), and IMI-D8 (Δ = 4.64 log) combinations, but re-

| TABLE 1 | Daptomycin and cell wall agent MICs for an isogenic pair of MRSA strains |
|-----------------------------------------------|
| **Strain** | **Daptomycin** | **Vancomycin** | **Ampicillin** | **Oxacillin** | **Cefazolin** | **Imipenem** | **Fosfomycin** | **D-Cycloserine** |
| A8796 | 0.5 | 1 | 16 | 512 | 128 | 64 | 2 | 32 |
| A8799 | 2 | 2 | 6 | 0.38 | 32 | 0.094 | 1 | 32 |

* 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 com-
binations 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
with D8 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\text{MIC}. Each culture
was then resuspended in fresh medium containing the positively
charged molecule cytochrome \text{c (CyC)} from bovine heart (Sigma).
The \text{A}_{405} 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 \text{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 sig-
nificant when \text{P values were} ≤0.05. In the absence of antibiotic
exposure, significant differences (\text{P < 0.05}) were found between
the amounts of CyC bound to strains A8796 (1.39 ± 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\text{MIC} was
significantly higher than that bound in its absence (1.26-fold in-
crease for A8796 and 1.68-fold increase for A8799). We hypothe-
size 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 in-
crease), which might be strain dependent. Also, since FOM inhib-
its the UDP-\text{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 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-

A: A8796 (DS)

B: A8799 (DNS)
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


