Daptomycin pharmacodynamics against Staphylococcus aureus hemB mutants displaying the small colony variant phenotype

Damir Begic, Christof von Eiff and Brian T. Tsuji

Laboratory for Antimicrobial Pharmacodynamics, School of Pharmacy and Pharmaceutical Sciences Buffalo and The New York State Center of Excellence in Bioinformatics and Life Sciences, University at Buffalo, Buffalo, NY 14260, USA; Institute of Medical Microbiology, University of Münster, Münster, Germany; Roswell Park Cancer Institute, Department of Medicine, Buffalo, NY 14263, USA

Received 5 September 2008; returned 12 December 2008; revised 6 February 2009; accepted 13 February 2009

Objectives: Staphylococcus aureus small colony variants (SCVs) are slow-growing morphological variants associated with persistent infections. While vancomycin activity has been shown to be attenuated against SCVs of S. aureus, few data exist regarding daptomycin. The objective was to evaluate the pharmacodynamics of daptomycin against defined S. aureus mutants displaying the SCV phenotype.

Methods: Two S. aureus hemB mutants (Ia48 and III33) displaying the SCV phenotype and their parental strains (COL and Newman) were evaluated. Time–kill experiments were performed using a starting inoculum of 10^6 cfu/mL at 0, 0.25, 0.5, 1, 2, 4, 8, 16, 32 and 64 times the MIC. Samples were obtained at 0, 1, 2, 4, 6, 8 and 24 h, plated and incubated to determine colony counts. A Hill-type pharmacodynamic mathematical model was fitted to the data to characterize the effect.

Results: Bactericidal activity for daptomycin was achieved and occurred in a concentration-dependent manner against both hemB mutants and their parental strains. Against strains with normal phenotype, bactericidal activity was achieved rapidly, within 2 h at concentrations ≥16 times the MIC, while against SCVs, bactericidal activity was achieved within 6 h at concentrations ≥16 times the MIC. Against both hemB mutants, daptomycin maintained bactericidal activity at 24 h, with similar profiles of killing activity when compared with their parental strains.

Conclusions: Daptomycin achieved bactericidal activity against S. aureus hemB mutants and parental isolates. Daptomycin represents a potential therapeutic option for infections caused by S. aureus strains displaying the SCV phenotype and additional studies are warranted.

Keywords: S. aureus, SCVs, cyclic lipopeptide antibiotics

Introduction

Staphylococcus aureus small colony variants (SCVs) are a slow-growing subpopulation associated with persistent, recurrent and antibiotic-refractory infections. These variants are described by distinctive phenotypic characteristics and pathogenic traits such as slow growth, decreased pigment formation, reduced haemolytic activity and decreased coagulase activity. In particular, infections caused by methicillin-resistant S. aureus (MRSA) SCVs are exceptionally difficult to treat. The ability of S. aureus SCVs to resist antimicrobial therapy may be due to their ability to persist and survive intracellularly in non-professional phagocytes without lysing the cells. Thus, the intracellular location may shield the SCVs from surrounding host defences and antimicrobial agents that have limited ability to accumulate within the cells.

Daptomycin is a cyclic lipopeptide antibiotic that is indicated for infections caused by MRSA. The mechanism of action of daptomycin is the disruption of the bacterial plasma membrane function without penetrating the cytoplasm, causing membrane depolarization and resulting in rapid cell death. Data from a previous study among isogenic strains of S. aureus displayed the normal and SCV phenotypes suggested that vancomycin killing activity is significantly attenuated against SCVs. Although these data provided insights into potential associations impacting vancomycin tolerance, pharmacodynamic data are limited.
among newer antimicrobial agents against staphylococcal SCVs. Therefore, as an extension from our vancomycin studies, the same isogenic strains displaying the normal and SCV phenotypes were utilized to examine the pharmacodynamics of daptomycin against \textit{S. aureus}.

## Materials and methods

### Bacterial strains

Bacterial strains utilized in this study included \textit{S. aureus} COL (MRSA) and Newman [methicillin-susceptible \textit{S. aureus} (MSSA)] and the respective site-directed \textit{hemB} mutants with SCV phenotype Ia48 (COL \textit{hemB::ermB}) and III33 (Newman \textit{hemB::ermB}), which were identical to the isogenic strains utilized in our previous study with vancomycin. The construction of both mutants, Ia48 and III33, by allelic replacement of the \textit{hemB} gene of \textit{S. aureus} strains COL and Newman has been previously described.

### Antibiotic, susceptibility testing and medium

Daptomycin analytical grade powder was obtained from Cubist Pharmaceuticals, Lexington, MA, USA. Fresh working solutions of daptomycin were made prior to each experimental run. MICs were determined in quadruplicate using a microdilution technique in accordance with the CLSI criteria. Mueller–Hinton broth (Difco, Detroit, MI, USA) supplemented with 50 mg/L calcium and 12.5 mg/L magnesium (SMHB) was used for all time–kill and susceptibility testing involving daptomycin.

### Time–kill experiments

Fresh bacterial colonies from an overnight growth were added to SMHB and adjusted spectrophotometrically to provide a standard suspension. This suspension was diluted with SMHB and a standard antibiotic stock solution to achieve a starting inoculum of \(10^8\text{ cfu/mL}\). The following concentrations were tested for daptomycin: 0, 0.25, 0.5, 1, 2, 4, 8, 16, 32 and 64 times the MIC. Each 20 mL culture was incubated in a water bath at 35°C with constant shaking, and samples were withdrawn for the determination of bacterial counts at 0, 1, 2, 4, 6, 8 and 24 h. Colony counts were determined by plating 100 \(\mu\text{L}\) of each diluted sample onto tryptic soy agar plates containing 5% sheep’s blood (Becton-Dickinson, Franklin, Lakes, NJ, USA) with an automated spiral dispenser (WASP; Don Whitley Scientific Limited, West Yorkshire, UK), incubating for 24 h at 35°C and then counting using an acolyte colony counter (Don Whiteley): these methods resulted in a limit of detection of 2 \(\log_{10}\) cfu/mL. Ia48 and III33 were incubated for 48 h. All time–kill experiments were completed in duplicate.

### Pharmacokinetic and pharmacodynamic analyses

To accommodate all available data generated for each regimen tested and avoid conclusions based on cfu counts at a single time-point, an integrated pharmacokinetic/pharmacodynamic area measure (log ratio area) was applied to all cfu data. For each regimen tested, the area under the cfu versus time curve from 0 to 24 h (AUcfu\(_{0-24}\)) was calculated via the trapezoidal rule for both growth control (AUcfu\(_{growth\ control}\)) and drug-containing regimens (AUcfu\(_{drug}\)). The AUcfu\(_{0-24}\) was normalized by the AUcfu\(_{0-24}\) of the growth control and the logarithm of this ratio was used to quantify the drug effect as shown in Equation (1). Additionally, the traditional approach (log ratio change) of comparing the changes in cfu/mL from 0 h (cfu\(_0\)) versus 24 h (cfu\(_{24}\)) was to calculate as shown in Equation (2).

\[
\text{log ratio area} = \log_{10}\left(\frac{\text{AUcfu}_{drug}}{\text{AUcfu}_{growth\ control}}\right) \quad (1)
\]

\[
\text{log ratio change} = \log_{10}\left(\frac{\text{cfu}_{24}}{\text{cfu}_{0}}\right) \quad (2)
\]

Using non-linear regression, a four-parameter concentration–effect Hill-type model was fitted to the effect parameter SigmaPlot (Version 11, Richmond, VA, USA) using:

\[
E = E_0 - \frac{E_{\text{max}} \times [\text{C:MIC}]^H}{[\text{EC}_{50}]^H + [\text{C:MIC}]^H} \quad (3)
\]

where the dependent variable (E) is either log ratio area or log ratio change, \(E_0\) is the measured effect at zero drug concentration, \(E_{\text{max}}\) is the maximal effect, \([\text{C:MIC}]\) is the concentration of drug divided by \([\text{MIC}]\), \([\text{EC}_{50}]\) is the C:MIC for which there is 50% maximal effect and \(H\) is the Hill or sigmoidicity constant.

## Results

Daptomycin displayed MICs of 1.0 mg/L for both \textit{S. aureus} strains COL and Newman and MICs of 2.0 mg/L for both \textit{hemB} mutants Ia48 and III33. Daptomycin exhibited concentration-dependent bactericidal activity against both \textit{S. aureus} strain pairs expressing normal and SCV phenotypes. With increasing daptomycin concentrations, a concentration-dependent trend towards greater bacterial killing was observed for both strains and phenotypes as shown in Figure 1. Against COL and Newman, exposure to daptomycin at concentrations \(\geq 4\) times the MIC achieved 99.9% kill in 6 h or less. In Ia48 and III33, 99.9% kill was achieved by daptomycin at concentrations \(\geq 4\) times the MIC in 6 h or less. Against COL and Newman, bactericidal activity was achieved rapidly, within 2 h at concentrations \(\geq 16\) times the MIC. Against Ia48 and III33, bactericidal activity was achieved within 6 h at concentrations \(\geq 16\) times the MIC. At early timepoints (\(\leq 2\) h), daptomycin resulted in a 5 log reduction against the normal phenotype strains versus approximately a 1 log reduction in cfu/mL against the SCV strains. Bactericidal activity was sustained at 24 h for all concentrations \(\geq 4\) times the MIC. Against both \textit{hemB} mutants displaying the SCV phenotype, daptomycin maintained bactericidal activity at 24 h, with similar profiles of killing activity when compared with their parent strains.

Model-fitted parameter estimates using Equation (3) and model fits characterizing the concentration–effect relationship for daptomycin against all strains are displayed in Figure 2. Analysis of pharmacodynamics revealed excellent model fits of the data to the Hill model. Among the two pharmacodynamic methods, the log ratio area approach, which accounted for all of the available data in 24 h, revealed better fits to the model,
as $R^2$ was $>0.99$ for all isolates. Bactericidal activity occurred for daptomycin in a concentration-dependent manner against both hemB mutants and their parental strains. Using the log ratio area approach, hemB mutants generally displayed a shallower concentration–effect profile with low Hill’s constant values of 1.02 and 1.87 for the hemB mutants Ia48 and III33, respectively, compared with their isogenic parent strains, which displayed the normal phenotype. However, the EC$_{50}$ and maximal effect for daptomycin, $E_{max}$, were similar among hemB mutants compared with parental strains.

**Discussion**

Daptomycin is a cyclic lipopeptide agent that exhibits rapid, concentration-dependent *in vitro* bactericidal activity against a variety of strains of Gram-positive pathogens.$^9$ Daptomycin has become an alternative treatment option for infections caused by MRSA; however, the pharmacodynamic profile against strains with SCV phenotype has not been fully elucidated. Interestingly, this is the first report that analyses the concentration–effect relationship of daptomycin with *S. aureus* SCVs. In previous experiments that we recently performed using isogenic strains of *S. aureus*, vancomycin killing of hemB mutants displaying the SCV phenotype demonstrated ~50% less effect than the vancomycin killing of their parental strains.$^6$ However, in the current study, against the identical isogenic isolates as the vancomycin experiments, daptomycin displayed sustained bactericidal activity against *S. aureus* strains that possessed the SCV phenotype. Interestingly, although early killing activity was attenuated, final bacterial endpoints and pharmacodynamics were similar when comparing hemB SCV mutants and normal
Figure 2. Pharmacodynamic relationship between daptomycin concentration to minimum inhibitory concentration ratio (C:MIC) and change in log10 cfu/mL at 24 h (log ratio change) against MRSA COL (a), Ia48 (c), MSSA Newman (e) and III33 (g), and area under the cfu curve of drug/area under the cfu curve of growth control (log ratio area) for MRSA COL (b), Ia48 (d), MSSA Newman (f) and III33 (h). N, normal phenotype; SCV, small colony variant phenotype; \( R^2 \), coefficient of determination. Data are reported as maximum likelihood model-fitted parameter estimates from Equation (3) for each strain.
phenotype *S. aureus* strains. These results are also in agreement with those of Baltch et al., who recently determined that daptomycin killing activity was similar against isogenic strains of MSSA Newman displaying the normal and SCV phenotypes: daptomycin decreased bacterial counts to nearly undetectable limits in both strains.

Two metabolic characteristics of *S. aureus hemB* mutants are believed to impact antimicrobial killing activity including their slow mode of growth and their reduced import of antimicrobials due to the decreased electrochemical gradient. The metabolic slow mode of growth and their reduced import of antimicrobials believed to impact antimicrobial killing activity including their organisms to persist within cultured endothelial cells. As daptomycin’s mechanism of action involves dissipation of *S. aureus* membrane potential, the interference of the electrochemical gradient may explain in part daptomycin’s retained killing activity against SCV mutants. Interestingly, in some daptomycin-resistant isolates, enhanced membrane fluidity, increased net positive surface charge, reduced susceptibility to daptomycin-induced depolarization and a significantly lower surface binding have been determined.

Additionally, previous studies have determined that *S. aureus* cell physiology may influence antimicrobial bactericidal activity. The mechanism of action of many bactericidal antibiotics requires continuous cell activity and cell division for the drugs’ killing activity. The SCV phenotype in *S. aureus* has also been associated with slow or stationary-phase growth. Mascio et al. determined that in high inoculum, stationary-phase cultures of *S. aureus*, daptomycin retained bactericidal activity but that elevated levels of the antibiotic were required to achieve the 3 log reduction endpoint. Interestingly, similar to the killing activity against stationary-phase organisms, against genetically defined mutants displaying SCV phenotype, daptomycin also achieved bactericidal activity at 24 h, although early killing activity was altered. Additionally, there was a trend towards greater overall killing by daptomycin of the SCVs versus parenteral isolates at concentrations closer to the MIC, although this trend was not maintained in the final pharmacokinetic/pharmacodynamic analyses. Taken together with our recent findings that vancomycin activity is markedly attenuated versus *hemB* SCV mutants, and in search of optimal therapeutic options against staphylococcal SCVs, these findings are of interest and highlight the need for additional therapeutic agents against SCV MRSA strains. Coupled with the ability of SCVs to persist intracellularly within the host and the antimicrobial-tolerant nature of SCVs, these findings may also suggest that early aggressive therapy to rapidly reduce large bacterial inocula may potentially be of use against SCVs implicated in persistent and difficult-to-treat MRSA infections. Studies directed towards defining the optimal dose intensity, timing and duration of antimicrobials to eradicate staphylococcal SCVs and additional *in vivo* studies are needed to confirm these findings before these results are translated to the clinic.

**Acknowledgements**

These results were presented at the Eighteenth European Congress of Clinical Microbiology and Infectious Diseases, Barcelona, 2008, Abstract C1-676.

We thank Alan Forrest for insight into PK/PD analyses and Pamela Kelchlin for excellent technical assistance.

**Funding**

This study was funded in part by an investigator-initiated grant from Cubist Pharmaceuticals Inc.

**Transparency declarations**

B. T. T. has received grant support through Cubist Pharmaceuticals, Lexington, MA, USA. C. v. E. is an employee of Wyeth Pharmaceuticals. D. B. has nothing to declare.

**References**


