Pharmacodynamic profile of daptomycin against Enterococcus species and methicillin-resistant Staphylococcus aureus in a murine thigh infection model

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Objective: To describe the pharmacodynamic profile of daptomycin against methicillin-resistant Staphylococcus aureus (MRSA) and Enterococci species based on bacterial density in an immunocompromised mouse thigh infection model.

Materials and methods: The pharmacodynamic (PD) profile of daptomycin was determined against two MRSA, one vancomycin-resistant Enterococcus faecium, and one vancomycin-susceptible Enterococcus faecalis using the immunocompromised murine thigh model. Efficacy was assessed by the change in log₁₀ cfu in thighs after 24 h of drug treatment.

Results: Daptomycin produced a maximal kill of 4.5–5 log₁₀ cfu against the MRSA and 1.5–2 log₁₀ for the Enterococcus species. AUC/MIC was the most predictive of the PD parameters. Utilizing MICs determined in serum or broth in the calculation of the PD parameters had minimal effect on this correlation. AUC⁰⁰/free/MICbroth required for static effects with MRSA and Enterococcus species were 12–36 and 5–13, whereas 99% of maximal kill was achieved at ratios of 171–442 and 38–157, respectively.

Conclusions: These data reveal the potent in vivo bactericidal activity of daptomycin against MRSA and Enterococcus species using clinically achievable drug exposures (dose 4–6 mg/kg per day) currently under investigation in man.

Keywords: pharmacodynamic profiles, lipopeptides, Gram-positive organisms

Introduction

The proper administration of antimicrobials in the clinical setting requires a thorough understanding of pharmacodynamics (PD). Complete assessment of a compound’s pharmacodynamic profile requires varying exposures, which are attained by altering both the dose and frequency of drug administration. Moreover, this range of drug exposures should be sufficient to produce an array of effectiveness based on the outcome parameter of interest (e.g. growth of bacteria in vivo in the presence of low ineffective doses up to maximal bacterial kill based on the limit of detection with high drug exposures). As a result of the implications in conducting such studies in humans, the murine thigh infection model has been successfully utilized to describe the pharmacodynamic profile of various antimicrobial agents.

Daptomycin is a novel lipopeptide antimicrobial agent, and is a natural fermentation product of Streptomyces roseosporus. It is a Gram-positive-specific agent, which functions by the rapid depolarization of the bacterial membrane leading to reduced DNA, RNA and protein synthesis.¹ It has in vitro activity against a wide variety of Gram-positive pathogens including methicillin-resistant Staphylococcus aureus (MRSA), vancomycin-resistant enterococci spp., and penicillin-non-susceptible Streptococcus pneumoniae.²,³ The activity of daptomycin is dependent upon physiological concentrations of calcium. The objective of this study was to examine the pharmacodynamic profile of daptomycin against clinical isolates of MRSA, and vancomycin-susceptible and vancomycin-resistant Enterococcus species.

Materials and methods

Antimicrobial test agents

Daptomycin (lot 701703A, purity 92.66%) supplied by Cubist Pharmaceuticals Inc. (Lexington, MA, USA) was utilized throughout this study.

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Daptomycin powder was reconstituted in sterile water based on mean animal weight and administered as a subcutaneous injection.

Bacterial isolates and susceptibilities

Four isolates were used in this study; two MRSA, MRSA 43300 (ATCC strain 43300) and MRSA 494 (Glenn W. Kaatz, Wayne State University, Detroit, MI, USA), one vancomycin-susceptible Enterococcus faecalis, VSEF-49452 (ATCC strain 49452; vancomycin MIC = 0.78 mg/L) and one vancomycin-resistant Enterococcus faecium, VREF 80 (EF80, Cubist Pharmaceuticals; vancomycin MIC > 100 mg/L).43 The minimum inhibitory concentrations (MICs) of daptomycin were determined using the microdilution method according to NCCLS guidelines using Cation-Adjusted Mueller–Hinton Broth (Becton-Dickinson and Company, Cockeysville, MD, USA) supplemented with [Ca]2+ for a final concentration of 50 mg/L.44 The MICs were determined 3–12 times and the median value was utilized in the pharmacodynamic (PD) analysis (MICserum). MIC tests were also carried out in 100% pooled female mouse serum. Serum MIC (MICserum) tests were repeated using different doubling dilutions in order to more closely estimate the serum MIC. Since two repeats of the serum MIC were conducted with different concentrations, the mean of these two values was used in the PD analysis (MICserum).

Thigh infection model

Female, specific-pathogen free ICR mice (Harlan Sprague–Dawley Inc.; Indianapolis, IN, USA) weighing approximately 25 g were used throughout the study. Mice were quarantined 7 days before use and were allowed food and water ad libitum throughout the study. Hartford Hospital (Hartford, CT, USA) Institutional Animal Care and Use Committee reviewed and approved the methodology for use of these animals. Mice were rendered neutropenic by a series of cyclophosphamide (Cytoxan, Bristol-Myers Squibb, Princeton, NJ, USA) injections given intraperitoneally 4 days and 1 day before daptomycin dosing utilizing a dose of 150 mg/kg and 100 mg/kg, respectively. This regimen was chosen based on prior studies, where neutropenia was maintained for 5 days.7,8 The study schematic is shown in Figure 1.

Isolates used for suspension were stored on blood agar slants, which were stored and subcultured once before use. The bacterial suspensions were prepared by bringing Mueller–Hinton broth to a turbidity equivalent to that of a 0.5 McFarland standard (Remel, Lenexa, KS, USA) (∼1.5 × 10^8 cfu/mL) with fresh growth. A 10-fold dilution was carried out on the ∼1.5 × 10^8 cfu/mL suspension for the MRSA isolates before inoculation; however, the bacterial suspensions of the Enterococcus species isolates were not subsequently diluted before animal inoculation. Unanaesthetized mice were inoculated with 0.1 mL into each of the two rear thighs; for statistical purposes, each thigh was considered to be an individual data point. Once inoculated, mice were randomized into the control or treatment groups.

Daptomycin dosing was initiated in the treatment groups 2 h after thigh inoculation. For each isolate eight different daptomycin regimens were chosen over a broad range of exposures. Dose frequency ranged from once daily (QD), twice daily (BID), three times daily (TID), and four times daily (QID). Doses were administered at hour 0 (QD, BID, TID, QID), hour 6 (TID, QID), hour 12 (BID, TID, QID), and hour 19 (QID). Control animals were concurrently administered saline (mock treatment) subcutaneously in the same volume and frequency as those receiving daptomycin. Treatment groups were dosed for 24 h then killed at hour 24. There were two control groups for each isolate where one was killed at the initiation of the dosing (2 h post-thigh inoculation) and the other at 24 h post the initiation of dosing. Mice were killed via inhalation of CO2 followed by cervical dislocation. Immediately following sacrifice, thigh tissue was collected from the animal and tissue was homogenized in 5 mL of saline. Thigh homogenate was then diluted with 10-fold serial dilutions and streaked onto growth medium in spirals using the Autoplate 4000 (Spiral Biotech, Norwood, MA, USA). Medium was incubated for 18–24 h at 35°C in ambient air. Using this methodology for streaking thigh homogenate, the lower limit of detection for in vivo studies was 50 cfu/thigh (i.e. 1.7 log_{10}cfu).

Pharmacokinetic studies

Preliminary pharmacokinetic (PK) studies were conducted by Cubist Pharmaceuticals. In these studies, non-neutropenic, uninfected, specific-pathogen free, female ICR mice were administered 10, 50 or 100 mg/kg of daptomycin by subcutaneous injection. Blood samples were obtained from three mice at each sampling time (0.25, 0.5, 1, 2, 3, 4, 5 and 6 h post-dosing). For each dose and drug concentrations were determined by a validated high performance liquid chromatography (HPLC) procedure as noted below. Based on the concentration–time profiles obtained, we completed the pharmacokinetic analysis using WinNonlin Pro, version 3.0 (Pharsight Corporation, Mountain View, CA, USA).

Confirmatory pharmacokinetic studies were done at Hartford Hospital to validate preliminary pharmacokinetic studies. However, unlike the initial studies, mice were rendered neutropenic and inoculated as in the manner previously reported. Daptomycin (50 mg/kg) was administered subcutaneously and blood samples were collected from six mice (at 1, 2, 4 and 6 h post-dosing).

The protein binding value of 90% in mice used in this analysis was obtained from published data.10 Given the linear nature of the protein binding over the range of doses used, free drug concentrations were employed in the calculation of the pharmacodynamic profile (e.g. AUC/MIC).
Pharmacodynamic profile of daptomycin

Analytical assay
Cubist Pharmaceuticals determined plasma concentrations of daptomycin in all studies. Daptomycin was detected using an internal standard (IS) of ethylparaben and protein precipitation followed by HPLC using methodology developed and validated by Cubist Pharmaceuticals (data on file with sponsor). In brief, drug concentrations were determined by reverse phase HPLC using a Metachem Hypersil C8 analytical column and a Waters Xterra RP18 guard column (ANSYS Technologies, Inc., Lake Forest, CA, USA). Daptomycin and IS were isolated using protein precipitation methods. The mobile phase was 32.6% acetonitrile and 67.4% of a 0.5% ammonium phosphate solution. At a flow rate of 1.5 mL/min, daptomycin shows a retention time of 14 to 16 min. Samples were analysed at 214 nm. The detection of daptomycin concentrations in plasma was linear across the range of 7.5–400 mg/L.

Data analysis
Efficacy was assessed by the change in log_{10} cfu of the treatment and 24 h control group from the zero hour control group (2 h after inoculation). The arithmetic mean of the change in log_{10} cfu for each treatment and control group was then utilized in the pharmacodynamic analysis.

Dose fractionation studies were undertaken to determine the PD parameter most closely related to outcome for the bacteria studied. Once the optimal parameter was determined, the full pharmacodynamic profile was delineated using the complete range of drug exposures (i.e. AUC\textsubscript{free}/MIC\textsubscript{broth}) with their corresponding efficacy values (i.e. change in log_{10} cfu) using the sigmoid E\textsubscript{max} model (WinNonlin Pro, version 3.0; Pharsight Corporation). From this profile the bacteriostatic, ED\textsubscript{80} and ED\textsubscript{99} exposures were derived. Bacteriostatic dose is defined as the exposure (in terms of the optimal parameter) whereby the log_{10} cfu at 24 h was equal to that at the initiation of dosing (i.e. zero hour control group) that is equivalent to a change in log_{10} cfu of 0. ED\textsubscript{80} and ED\textsubscript{99} are defined as the exposures (in terms of the optimal parameter) where 80% and 99% of the maximal kill are seen.

Additional analysis was conducted to evaluate different methods of incorporating protein binding in calculations of the pharmacodynamic profile as follows: protein binding was incorporated either by using free drug exposures with broth MIC (AUC\textsubscript{free}/MIC\textsubscript{broth}) or total drug exposures and MICs as determined in serum (AUC\textsubscript{total}/MIC\textsubscript{serum}).

Results

Susceptibility studies
The median MIC values for the MIC\textsubscript{broth} are listed in Table 1. The arithmetic mean of the serum MIC was used in the pharmacodynamic calculations of MIC\textsubscript{serum} (Table 1). [Ca\textsuperscript{2+}] content in mouse serum was found to fall within the range of human physiological [Ca\textsuperscript{2+}]. Daptomycin MIC values were 13–18 times higher in 100% mouse serum than in broth.

Pharmacokinetic studies
The plasma concentration–time profile including fitted plots for three doses are displayed in Figure 2. Pharmacokinetic analysis was conducted using a one-compartment model with first-order absorption and elimination with a lag time. Over the dose ranges used, linearity was observed (10, 50 and 100 mg/kg). The mean pharmacokinetic parameters from the three doses were determined to have a half-life of 1.45 h, volume of distribution of 5.12 mL, and absorption rate constant (k\textsubscript{a}) of 20.48. In the confirmational PK study (Figure 3), the concentrations at the time points noted were shown to have no significant difference (P > 0.05) from those obtained in the original PK profile of the compound. As a result, the PK parameters derived from these studies were used to simulate the concentration–time profile of different dosing regimens used in the bacterial density studies.

Table 1. MIC\textsuperscript{a} determined in broth and murine serum

<table>
<thead>
<tr>
<th>Isolate</th>
<th>MIC\textsubscript{broth} (mg/L)</th>
<th>MIC\textsubscript{serum} (mg/L)\textsuperscript{d}</th>
</tr>
</thead>
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<tr>
<td>MRSA 43300\textsuperscript{b}</td>
<td>0.25</td>
<td>4.5</td>
</tr>
<tr>
<td>MRSA 494\textsuperscript{b}</td>
<td>0.25</td>
<td>4.5</td>
</tr>
<tr>
<td>VSEF-49452\textsuperscript{b}</td>
<td>1.0</td>
<td>13</td>
</tr>
<tr>
<td>VREF 80\textsuperscript{b}</td>
<td>2.0</td>
<td>36</td>
</tr>
</tbody>
</table>

\textsuperscript{a}Median of 3–12 determinations (broth) and mean of two determinations (serum).

\textsuperscript{b}Isolate obtained from Cubist Pharmaceuticals.

\textsuperscript{c}Isolate obtained from G.W. Katz.

\textsuperscript{d}Tested in 100% mouse serum.

Figure 2. Plasma concentration–time profile of daptomycin in normal mice.

Figure 3. Confirmatory pharmacokinetic studies with 50 mg/kg of daptomycin.
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Bacterial density studies

Bacterial suspensions prepared for infection of mice with MRSA isolates ranged from $1.6 \times 10^7$ to $2.0 \times 10^7$ cfu/mL and $7.2 \times 10^7$ to $1.47 \times 10^8$ cfu/mL for the *Enterococcus* species. In preliminary studies, *Enterococcus* species were found to require a higher inoculum in order to see minimal to increased growth of organism over a 24-h period. The bacterial recovery of thighs in the control groups at the initiation of dosing (2 h post thigh-infection) and after 24 h is displayed in Figure 4. Bacterial recovery in control mice 24 h after the initiation of dosing was found to have increased 2.23, 2.37, 1.03, and 0.24, respectively. The bacterial kill achieved is displayed in Figure 4 and ranged from 1.6 to 2.0 log units.

Thigh infection was effectively established in all four isolates and all but VREF 80 grew over a 24-h period. Control groups for isolates used in the dose fractionation studies (MRSA 494 and VSEF 49452) showed similar growth assessed by an $t$-test with $P > 0.05$.

In order to closely evaluate which PD parameter best correlates with efficacy, dose fractionation studies were undertaken to best define the PD parameter with which efficacy is best correlated. Results from dose fractionation studies are listed in Table 2. One-way ANOVA with Scheffe’s method (SPSS for Windows, version 10.1; SPSS Inc., Chicago, IL, USA) showed no significant difference ($P > 0.05$) between the log$_{10}$ cfu of each dose fractionation except for one (10 mg/kg QD; MRSA 494), suggesting that the AUC/MIC is most closely related to efficacy.

Correlations of bacterial kill and this pharmacodynamic parameter were assessed for each individual isolate. Figure 5 reveals the pharmacodynamic profile resulting from the plot of the AUC$_{free}$/MIC$_{bactericidial}$ and change in log$_{10}$ cfu/mL and their respective coefficients of determination ($r^2$) for all four isolates.

Maximum bacterial kill achieved for each isolate was assessed by the change in log$_{10}$ cfu between the 24-h control and the maximum bacterial kill achieved. The maximum bacterial kill, or maximum effectiveness for each isolate is 4.45, 5.03, 2.09 and 1.53 for MRSA 43300, MRSA 494, VSEF 49452 and VREF 80, respectively. A significantly greater amount of kill was observed with the MRSA isolates versus the *Enterococcus* species isolates ($P < 0.05$). The AUC/MIC ratios that produced bacteriostasis, 80% of maximum effectiveness (ED$_{80}$), and 99% of maximum effectiveness (ED$_{99}$) for each isolate, based on $E_{max}$ curve fit, are listed in Table 3.

Utilizing either determinant of exposure (i.e. AUC$_{free}$/MIC$_{bactericidial}$ or AUC$_{total}$/MIC$_{serum}$), the rate and extent of bactericidal activity were similar (Table 3) when the variability commonly associated with the MIC ($\pm 1$ two-fold dilution) is taken into account. These data indicate that using either method to assess the impact of protein binding would be accurate (Figure 6).

Discussion

Daptomycin is a novel antimicrobial that has demonstrated considerable *in vitro* activity against a wide range of Gram-positive pathogens. Whereas potent *in vitro* activity is often a prerequisite for the development of clinically viable compounds, the importance of an agent’s pharmacodynamic profile in the selection of clinical relevant exposures to ensure antimicrobial efficacy is now well recognized. As such, the goal of pharmacodynamic studies initially conducted in animal models of infection is to define both the parameter most predictive of efficacy ($C_{max}$/MIC, %T>MIC or AUC/MIC) and also the magnitude of this effect, which is required to optimize antibacterial or survival outcomes.

Our current study with daptomycin extends the observations noted by Louie et al., using a single isolate of *S. aureus*.10 These investigators evaluated the pharmacodynamic profile of daptomycin against *S. aureus* ATCC 29213, a methicillin-susceptible *S. aureus* (MSSA), in a similarly conducted neutropenic murine thigh model. Consistent with this report using MSSA, we also noted excellent *in vivo* bactericidal activity (>4 log$_{10}$ kill) with both of the methicillin-resistant *S. aureus* isolates used in this study. Moreover, we also determined that the AUC/MIC ratio was the pharmacodynamic parameter most predictive of this compound’s antibacterial efficacy for this species as assessed by the reduction in bacterial density over 24 h of exposure. Additionally, when one accounts for the potential variability in the determination of AUC/MIC ratios as assessed by a range of exposures, the bacteriostatic and ED$_{99}$ values determined using the AUC$_{total}$/MIC$_{serum}$ profile were similar between the two studies.

Safdar et al.11 also evaluated *S. aureus* (including MRSA) and *S. pneumoniae* (including penicillin-intermediate- and -resistant strains) utilizing the thigh infection model. In this study, the authors determined that the correlation of efficacy, in terms of bacterial killing, was related to both $C_{max}$/MIC and AUC/MIC.11 Whereas these authors determined the required exposures to produce a net bacteriostatic effect for *S. aureus* in terms of the AUC$_{free}$/MIC, this value was in the range of that observed in our current study. One of the staphylococcus isolates used in the current investigation, MRSA 494, has been previously studied both using an *in vitro* pharmacodynamic model and *in vivo* in the endocarditis model.4,5 In an *in vitro* model with simulated endocardial vegetation, daptomycin produced approximately a 6.5 log$_{10}$ kill of MRSA 494. The AUC/MIC ratio in this *in vitro* study was greater than 1000 for both regimens studied. In a rabbit model, daptomycin also demonstrated >6 log$_{10}$ kill in endocardial vegetations. In our *in vivo* murine thigh infection model, this same isolate demonstrated an approximately 5 log$_{10}$ kill (Figure 5) at a much lower AUC/MIC ratio (maximum ratio studied was 300).

The *in vitro* study by Akins & Rybak reported a daptomycin MIC$_{bactericidal}$ for MRSA 494 of 0.25 mg/L, which is utilized in our analy-
Pharmacodynamic profile of daptomycin

Table 2. Daptomycin dose fractionation studies for MRSA 494 and VSEF 49452

<table>
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<tr>
<th>Dose (mg/kg)</th>
<th>Frequency</th>
<th>Total daily dose (mg/kg)</th>
<th>AUC&lt;sub&gt;free&lt;/sub&gt;/MIC</th>
<th>C&lt;sub&gt;max&lt;/sub&gt;/MIC (free)</th>
<th>%T&gt;MIC</th>
<th>Change in log&lt;sub&gt;10&lt;/sub&gt; cfu/mL</th>
<th>Standard deviation</th>
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*QD = 0 h; BID = 0 and 12 h; TID = 0, 6 and 12 h; QID = 0, 6, 12 and 19 h.*

*A statistically significant difference (P < 0.05) was found between 10 mg/kg QD and the two fractionations (5 mg/kg BID and 2.5 mg/kg QID) using a one-way ANOVA with Scheffe’s method.

Figure 5. Pharmacodynamic profile of daptomycin as predicted by AUC<sub>free</sub>/MIC<sub>broth</sub> ratio.

In our assessment, the median MIC<sub>broth</sub> was found to be 0.5 mg/L, where nine of 11 repeats had a value of 0.5 mg/L. This difference may be due to the difference in [Ca]<sup>2+</sup> added to the broth used in the assessment of MIC. Whereas our laboratory supplements the broth to a calcium concentration of 50 mg/mL, Akins & Rybak supplemented broth to a calcium concentration of 75 mg/mL in order to maintain physiological calcium (50 mg/dL) in their in vitro model throughout their experiment. It has been well established that the mechanism of action of daptomycin is dependent on the presence of calcium. Quantitatively, this is seen whereby increasing concentrations of calcium lower the MIC for the organism. In a study by Petersen et al., a 2–3 two-fold dilution difference in MIC<sub>90</sub> was observed when broth calcium concentrations were 25, 50 and 75 mg/dL. Whereas a 1 two-fold dilution difference is an acceptable deviation from a microbiological perspective in the calculation of the pharmacodynamic parameters, this can translate into a 100% deviation. However, it is also important to note that at clinically achievable AUCs, calculations utilizing either MIC value predict no less than 80% bacterial kill.

Daptomycin has been shown in vitro to be active against Enterococcus spp. In vitro studies demonstrate >3 log<sub>10</sub> kill which is higher than the 1.5–2 log<sub>10</sub> kill observed in our current in vivo study (Figure 5). Previous studies utilizing S. aureus and S. pneumoniae indicate that bacterial killing efficacy does not vary amongst resistance mechanisms within these bacterial species. However, in the examination of Enterococcus spp., VREF 80 appeared to have a pharmacodynamic profile distinct from the other isolates studied, where maximal kill was achieved at lower AUC/MIC ratios as indicated in Table 3 and depicted in Figure 5. Whereas its bacterial killing activity is distinct, it is important to also consider that whereas VREF 80 infection was established in animals, the 24-h growth was negligible, as indicated by untreated control groups. Whereas a clear reason for
this distinct PD profile is not readily apparent, it may be due to the difference in resistance mechanisms or virulence factors associated with this isolate.

Daptomycin is approximately 90% bound by serum proteins with values similar in both murine and human sera. Throughout the study, protein binding was considered in the calculation of the PD parameters. In using AUCfree/MICbroth, free drug exposures were utilized in the calculations and in AUCtotal/MICserum, protein binding was accounted for by the serum MIC. As might be expected from the protein binding data with the compound, serum-derived MICs were found to be 92–94% of the corresponding broth MIC. Whereas the conventional thought dictates that for highly protein-bound drugs (>80%) a 4–5% deviation in this ‘apparent protein binding’ may translate into 50% less active drug, which would in turn affect the efficacy, Figure 6 shows that this is in fact not what is seen. Both the AUC/MIC curves utilizing either the broth or serum MIC are in general within one standard deviation of the observed bacterial killing profile.

Pharmacodynamics has played an integral role in the dosing of daptomycin. Original clinical trials in the late 1980s–early 1990s utilized 2–3 mg/kg doses given every 12 h. A more thorough understanding of the pharmacodynamics, including the current evaluation shows that a 4 or 6 mg/kg once daily dose (steady-state AUCtotal values obtained are 494 mg·h/L for 4 mg/kg and 747 mg·h/L for 6 mg/kg, data on file Cubist Pharmaceuticals) of daptomycin provides sufficient drug exposures to produce a reliable killing profile for both MRSA and enterococci. Moreover, when MIC90 of daptomycin for MRSA, VSEF and VREF of 0.5, 2.0 and 4.0 mg/L are incorporated into our derived pharmacodynamic profile (e.g. AUCfree/MIC) for the compound, this more conservative assessment also predicts bacterial kill in vivo for both MRSA and Enterococcus species when doses of 4–6 mg/kg are administered once-daily in man.2,3

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