Daptomycin-reversible rifampicin resistance in vancomycin-resistant Enterococcus faecium

Kenneth H. Rand1*, Herbert J. Houck1 and Jared A. Silverman2

1Department of Pathology, Immunology and Laboratory Medicine, University of Florida, Gainesville, FL 32610, USA; 2Cubist Pharmaceuticals, Lexington, MA 02421, USA

Received 20 October 2006; returned 20 November 2006; revised 12 January 2007; accepted 22 January 2007

Objectives: In a previous study, we observed marked synergy between daptomycin and rifampicin against 73% of rifampicin-resistant, vancomycin-resistant Enterococcus faecium (VRE), with approximately 100-fold reductions in rifampicin MICs observed at one-eighth to one-fourth daptomycin MIC. The purpose of this study was to determine whether the synergy between daptomycin and rifampicin could be explained by enhanced entry of rifampicin into the cell or was related to amino acid substitutions in the rifampicin-binding site in the β subunit (rpoB) of the RNA polymerase.

Methods: We developed a bioassay for rifampicin to measure cell-bound rifampicin levels as well as metabolic inactivation of rifampicin. In addition, we sequenced the rifampicin-binding site in the rpoB of VRE strains with and without synergy between daptomycin and rifampicin.

Results: Cell-bound rifampicin levels were the same in rifampicin-susceptible VRE as in rifampicin-resistant VRE showing daptomycin synergy and were not affected by the presence of daptomycin. In contrast, rifampicin-resistant VRE without daptomycin synergy had undetectable cell-bound rifampicin. Sequencing the rpoB rifampicin-binding site revealed that the synergistic strains had the same sequence as rifampicin-susceptible wild-type E. faecium. The daptomycin synergy-resistant strains all had mutations in known rifampicin-binding sites.

Conclusions: Daptomycin is able to reverse rifampicin resistance in some strains of VRE, but the mechanism could not be explained by an effect of daptomycin on entry of rifampicin into or transport out of the cell, by inactivation of rifampicin or by mutation involving the rifampicin-binding site.

Keywords: rpoB, rifampicin-binding site, E. faecium, VRE

Introduction

Daptomycin is a cyclic lipopeptide active against a wide range of Gram-positive bacteria, including problematic organisms such as vancomycin-resistant Enterococcus faecium (VRE). Daptomycin is believed to act at the cell membrane by insertion of its lipophilic ‘tail’, ultimately leading to the leakage of ions and cell death.1 In view of the relatively limited therapeutic options for VRE, a better understanding of the mechanism of action of effective antibiotics is important. We observed synergy between daptomycin and rifampicin for 73% of VRE with about 100-fold reductions in rifampicin MIC at one-fourth MIC daptomycin.2 In the current study, we extended the previous work to investigate the possible mechanisms of the synergy.

Rifampicin acts by entering the cell cytoplasm, binding to RNA polymerase (rpoB) and inhibiting transcription. Resistance to rifampicin is typically due to amino acid substitutions in the three known resistance clusters in the rifampicin-binding site of rpoB,3 but can also be mediated by efflux mechanisms or by enzymatic inactivation of the drug.4–7 Synergy between daptomycin and rifampicin against rifampicin-resistant VRE implies that daptomycin can overcome or reverse the mechanism of resistance in these isolates. Cell-bound rifampicin has been measured using [14C]rifampicin and shows rapid accumulation and intracellular binding in both Escherichia coli and Staphylococcus aureus.8 We used the procedure described by Williams and Piddock8 to measure cell-bound rifampicin, except that we developed a bioassay because radiolabelled rifampicin was not available. If binding site mutations are present, we expected to find little or no cell-bound rifampicin. The purpose of this study was to determine whether daptomycin affected levels of cell-bound rifampicin, which together with the binding
Materials and methods

Twelve strains of \textit{E. faecium} (vancomycin MIC $\geq 256$ mg/L, by Etest, AB Biodisk, Solna, Sweden) were obtained from the clinical microbiology laboratory at the Shands at the University of Florida Hospital, as previously described.$^4$ Four strains were rifampicin-susceptible, whereas eight were rifampicin-resistant (MIC $\geq 32$ mg/L by Etest). Of the eight rifampicin-resistant isolates, four displayed synergy with daptomycin (synergy-positive) and four did not (synergy-negative). Daptomycin MICs by broth dilution in Ca$^{++}$-supplemented (50 mg/L) Mueller–Hinton broth were 4 mg/L for all strains, except strain 11 (2 mg/L). Strain typing by PFGE at the Mayo Medical Laboratories (Rochester, MN, USA) showed that all strains were distinguishable from one another, except strains 10 and 11.

A bioassay for rifampicin was modified from that described by Dabbs$^5$ using a highly susceptible indicator strain of coagulase-negative \textit{Staphylococcus}. For a standard curve, Ca$^{++}$-supplemented (50 mg/L) Mueller–Hinton agar was inoculated with the indicator strain, and a series of 4 mm holes punched in the agar. The holes were then filled with rifampicin standards, 25 mg/L well containing 30, 7.5, 1.875 and 0.45 ng of rifampicin. Zone diameters were recorded after 18–24 h at 34°C.$^2$ Volumes (10 mL) of increased by using 15 mL of agar per 100 mm plate, instead of recorded after 18–24 h at 34°C. Strain 11 (2 mg/L). Strain typing by PFGE at the Mayo Medical Laboratories (Rochester, MN, USA) showed that all strains were distinguishable from one another, except strains 10 and 11.

\textit{Results}

The rifampicin bioassay was linear between 2 and 30 ng (data not shown). When cell-bound rifampicin was measured for synergy-positive strains (Table 1), an average of $\sim 5$ ng of rifampicin/10$^9$ cfu was detected. This value did not change in the presence of daptomycin up to 1 mg/L. A range of rifampicin values (0.9–13 ng/10$^9$ cfu) was observed for the different isolates tested, but in all cases, addition of daptomycin had no significant effect on rifampicin levels. In contrast, no cell-bound rifampicin ($<2$ ng/10$^9$ cfu) was observed for synergy-negative strains. This observation suggests fundamental differences in the mechanism of rifampicin resistance in synergy-positive and synergy-negative strains. Furthermore, since rifampicin-resistant strains had cell-bound rifampicin in the same range as that of the synergy-positive strains (data not shown), rifampicin efflux is unlikely to be responsible for resistance in synergy-positive strains.

Bacteria resistant to rifampicin frequently have mutations involving the rifampicin-binding site on the $\beta$ subunit of RNA polymerase.$^3$ Therefore, the $\rho\beta$ subunit of RNA polymerase was sequenced. Figure 1 shows that synergy-negative strains 1, 7 and 8 had an R529S substitution at a position known to interact with rifampicin in resistance Cluster I$^5$ and an additional amino acid substitution S574R in resistance Cluster II. Synergy-negative strain 11 had an L533F substitution at a position known to interact with rifampicin in Cluster I, as well as a G522D substitution in Cluster I. Strain 10 also had the L533F substitution, despite rifampicin susceptibility (MIC = 0.094 mg/L). No other significant differences were found. In contrast, synergy-positive strains had no amino acid differences from the reference \textit{E. faecium} strain 343-3 (GenBankAY167138),$^5$ strongly suggesting differences in the mechanism of resistance.

It was possible that the rifampicin-resistant VRE without $\rho\beta$ mutations were resistant to rifampicin because it was able to inactivate it. Several studies have demonstrated that species such as \textit{Mycobacterium avium}, \textit{Mycobacterium intracellulare}, \textit{Mycobacterium chelonae}, \textit{Mycobacterium fortuitum}, \textit{Nocardia}, \textit{Gordona} and \textit{Tsukamurella} spp. can inactivate rifampicin primarily by ribosylation.$^4$–$^7$ We tested the ability of the four synergy-positive VRE strains to inactivate rifampicin using the bioassay. VRE (10$^7$ log phase) were incubated with 2 mg/L of rifampicin for 24 h at 37°C. After incubation, bacteria were removed from the turbid suspension by centrifugation and 25 mg/L

\begin{table}[h]
\centering
\caption{Cell-bound rifampicin in VRE versus synergy between rifampicin and daptomycin}
\begin{tabular}{lll}
\hline
Daptomycin synergy & VRE strain no. & Rifampicin (ng/10$^9$ cells) \\
\hline
Present & 2 & 2.2$^b$ 0.9 \\
Present & 9 & 2.4 2.4 \\
Present & 12 & 13.0 11.2 \\
Present & 17 & 4.4 4.4 \\
Mean $\pm$ SD & & 5.5 $\pm$ 5.1 4.7 $\pm$ 4.5 \\
Absent & 1 & not detected not detected \\
Absent & 7 & not detected not detected \\
Absent & 8 & not detected not detected \\
Absent & 11 & not detected not detected \\
\hline
\end{tabular}
\end{table}

$^a$Daptomycin agar MIC = 2 mg/L for VRE strains 2, 9 and 12; MIC = 1 mg/L for VRE strain 17.

$^b$Rifampicin zones were interpolated from the standard curve within each experiment and normalized per 10$^9$ cells.
of the supernatant was assayed for rifampicin as described. Rifampicin concentrations in the supernatant from the synergy-positive strains were identical to that of broth alone incubated under the same conditions, suggesting that no rifampicin inactivation was occurring.

**Discussion**

Our findings indicate that the daptomycin synergy-positive, rifampicin-resistant VRE differ from the daptomycin synergy-negative strains in several important ways: first, they are capable of binding rifampicin, whereas the synergy-negative strains do not; secondly, the rifampicin-binding site is the same as that of susceptible VRE, whereas the synergy-resistant VRE have mutations in known resistance clusters and thirdly, daptomycin can reverse the rifampicin resistance in the synergy-positive strains, but not in the synergy-resistant strains.2 We were unable to demonstrate inactivation of rifampicin by any of our VRE, even after incubating strains for 24 h under conditions in which growth had become completely turbid. Since the daptomycin synergy-positive strains are phenotypically resistant to rifampicin (MIC > 32 mg/L),2 there must be another mechanism of rifampicin resistance besides mutation in the rpoβ gene and rifampicin inactivation.

We suggest that the mechanism of this novel rifampicin resistance in the daptomycin synergy-positive strains of VRE be termed daptomycin-reversible resistance, until the explanation for the reversal is worked out.

**Acknowledgements**

We gratefully acknowledge the support of the staff of the Shands at the University of Florida Hospital Clinical Microbiology Laboratory. This work is supported in part by a grant from Cubist Pharmaceuticals (Lexington, MA, USA) and by the Department of Pathology, Immunology and Laboratory Medicine, College of Medicine, University of Florida, Gainesville, FL, USA.

**Transparency declarations**

J. A. S. is employed by Cubist Pharmaceuticals. K. H. R. and H. J. H. have nothing to declare.

**References**

7. Quan S, Venter H, Dabbs ER. Ribosylative inactivation of rifampin by Mycobacterium smegmatis is a principal contributor to its low susceptibility to this antibiotic. Antimicrob Agents Chemother 1997; 41: 2456–60.