NOTES

Activities of Daptomycin and Comparative Antimicrobials, Singly and in Combination, against Extracellular and Intracellular Staphylococcus aureus and Its Stable Small-Colony Variant in Human Monocyte-Derived Macrophages and in Broth

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Received 14 November 2007/Returned for modification 4 December 2007/Accepted 25 February 2008

We investigated the antistaphylococcal activities of daptomycin, gentamicin, and rifampin against two Staphylococcus aureus strains and their stable small-colony variants, singly and in combination, in human monocyte-derived macrophages and in broth. Intracellularly, the three-drug combination and two-drug combinations with rifampin were most effective. Extracellularly, daptomycin, daptomycin plus gentamicin, gentamicin plus rifampin, and the three-drug combination had similar activities.

Infections caused by Staphylococcus aureus can be difficult to treat, and they persist and recur (5, 6, 16, 17). The intracellular survival of this bacterium and its ability to produce small-colony variants (SCVs) are thought to be responsible for these phenomena (15–17). SCVs grow slowly, remain intracellular, lack pigmentation and hemolytic activity, have reduced coagulase activity and alpha-toxin production, and have altered carbohydrate utilization (16, 17). Most clinical isolates are auxotrophic for menadione and hemin, two substances important in the biosynthesis of the electron transport chain elements menaquinone and cytochromes (15). This defect in the electron transport system permits SCVs to persist intracellularly (25). SCVs also resist antibiotic activity (5, 8, 13, 18). Daptomycin is a cyclic lipopeptide with rapid bactericidal activity against nonmultiplying gram-positive intracellular microorganisms at high cell densities (7, 10, 12, 21). Its intracellular activity has been demonstrated in neutrophils and monocytes (3, 23). Its mode of action is related to the rapid depolarization of the bacterial cell membrane and results in bacterial death without lysis (12, 21). Infections caused by SCVs have been described to occur in blood, bronchial secretions of cystic fibrosis patients, bones, and soft tissues and are associated with foreign material and prostheses (1, 6, 9, 19, 20, 24).

The purpose of this study was to investigate the antimicrobial activities of daptomycin, gentamicin, and rifampin, alone and in two- and three-drug combinations, against two S. aureus strains and their stable SCVs in human monocyte-derived macrophages (MDM). Results of intracellular experiments were compared with those from similar extracellular experiments with Mueller-Hinton II (MH-II) broth.

MICs (Table 1) were determined using standard CLSI (formerly NCCLS) methods (14). Two strains of S. aureus (SH 1000 [2546] and Newman [2548]) and their SCVs (DB27 [2547] and the Newman mutant [2549]) were obtained from Richard Proctor, University of Wisconsin. The SCVs were derived by insertion mutation and are genetically stable (25). Pulsed-field gel electrophoresis DNA fingerprinting of all four strains was performed as previously described (22). Parent strains and their corresponding SCVs were indistinguishable using this method (Fig. 1) and were assumed to be isogenic except for the insertion mutation mentioned above. In order that the effects of single drugs would not obscure those of drug combinations, concentrations of drugs that had antimicrobial activity but did not eliminate viable organisms in 24 h were used. Concentrations were 1× MIC for daptomycin (final Ca2+ concentration of 50 mg/liter) and 0.5× MIC for gentamicin and rifampin. The preparation of human monocytes (a consent form was approved by the Institutional Review Board of the Stratton VA Medical Center) and intracellular and extracellular assay procedures were as previously described (3). For data analysis, the analysis of variance method was used. The level of significance was 0.01. For time-kill experiments, inhibition is defined as the number of viable bacteria being statistically significantly less than the number in the untreated control but not statistically significantly less than the number at 0 h; killing is defined as the number of viable

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† Published ahead of print on 10 March 2008.

FIG. 1. PFGE DNA fingerprinting of S. aureus parent strains 2546 and 2548 and the SVC 2547 and SCV 2549 strains (22).
bacteria being statistically significantly lower than the number at 0 h. All experiments were performed in duplicate three times.

Differential fluorescent staining of intracellular and extracellular bacteria was done as described previously (2, 11), with modifications for *S. aureus*. Extracellular bacteria were incubated with polyclonal anti-*S. aureus* primary antibody and then stained with Alexa 488 (yellow-green) secondary antibody. MDM were then permeabilized, reincubated with anti-*S. aureus* antibody, and stained with Alexa 568 (red) antibody. DAPI (4',6-diamidino-2-phenylindole) stain (blue) was used.
for nuclei. Figure 2 shows clusters of intracellular \textit{S. aureus} microorganisms, with occasional extracellular microorganisms. Greater than 85\% of \textit{S. aureus} parent strain bacteria (Fig. 2A) and their SCVs (Fig. 2B) were intracellular in MDM assays. Occasional extracellular bacteria were seen (Fig. 2A to C).

Drug susceptibilities are shown in Table 1. Using CLSI criteria (14), all strains except SCV 2549 (resistant to gentamicin) were susceptible to the antibiotics tested. Susceptibilities of the SCVs to gentamicin were 1 dilution higher than those of their parent strains.

Results of intracellular experiments with MDM indicated that all single drugs and their combinations reduced the numbers of viable bacteria of both parent strains at 4 h ($P < 0.01$) (Fig. 3A and C). For parent strain 2548, gentamicin and rifampin were less active ($P < 0.01$) than daptomycin, the activity of which was similar to that of the drug combinations. At 24 and 48 h, two-drug combinations containing rifampin were more effective ($P < 0.01$) than gentamicin plus daptomycin for all strains except the \textit{S. aureus} parent 2548 at 48 h (Fig. 3C). The three-drug combination was most effective ($P < 0.01$) at

FIG. 4. Extracellular effects of single drugs and two- and three-drug combinations against the \textit{S. aureus} parent strain 2546 (A) and SCV 2547 (B), the parent strain 2548 (C), and SCV 2549 (D) in MH-II broth. Dotted lines represent results for single drugs. Numbers of viable bacteria at 0 h were $1 \times 10^6$ to $2 \times 10^5$ CFU/ml. Prior to sampling at 0, 4, 24, and 48 h, broth cultures were thoroughly mixed by vortexing. Samples were then taken, and viable bacteria in the samples were enumerated using the standard plate count method. The limit of detection was 10 CFU/ml. Results are from three independent experiments, each done in duplicate. Error bars represent standard errors of the means (some error bars are obscured by the symbols). Dap, daptomycin; Gent, gentamicin; Rif, rifampin.
24 and 48 h for all *S. aureus* strains, with the exception of SCV 2549, for which gentamicin plus rifampin was most effective at 48 h (*P < 0.01*). It is interesting to note that in MDM in the absence of antibiotics, one SCV (SCV 2547) grew more slowly than the other and did not attain the same cell density in MDM as its parent strain (*P < 0.01*). The slower intracellular growth of the SCVs may be an important factor in their reduced intracellular antibiotic susceptibilities.

Results of extracellular experiments in MH-II broth indicated that at 4 h, daptomycin and gentamicin were the most effective single drugs for both the *S. aureus* parent strains and SCV 2549 (*P < 0.01*) (Fig. 4A, B, and D). Daptomycin plus gentamicin was the most effective drug combination at 4 h (*P < 0.01*). However, at 24 and 48 h, the three-drug combination was most effective (*P < 0.01*), with activity similar to that of daptomycin plus gentamicin or gentamicin plus rifampin, and it was most effective against all strains except SCV 2549, for which daptomycin alone was most effective (Fig. 4D). At 48 h, treatment with daptomycin alone and all drug combinations was very effective, resulting in very low viable-cell counts (≤10 CFU/ml).

In most cases, we have observed reduced intracellular antibacterial activity in MDM compared to that seen extracellularly in MH-II broth. These results are similar to those described previously for MDM and other *S. aureus* strains (3) and by Barcia-Makay et al., who used THP-1 macrophages (4). However, the interpretation of differences between intracellular and extracellular activities is complex, since the intracellular activities of the antibiotics depend upon many factors, including their external concentrations, the times of exposure, and the pharmacokinetics of the drug (4).

In conclusion, we found the most effective (*P < 0.01*) intracellular drug(s) in MDM to be rifampin or rifampin in combination with daptomycin and/or gentamicin. In contrast, the most effective (*P < 0.01*) drug extracellularly in MH-II broth was daptomycin alone or daptomycin in combination with gentamicin. Our studies indicate that the three-drug combination or appropriate two-drug combinations that include rifampin are more effective (*P < 0.01*) intracellularly than single drugs against both parent *S. aureus* strains and their SCVs. Extracellular studies with MH-II broth, however, demonstrate similar levels of effectiveness for daptomycin alone or in combination with gentamicin, with or without rifampin, against both parent strains and SCV 2547 (*P < 0.01*). Both intracellular and extracellular activities are important because *S. aureus* strains are likely to be present in both locations during infections. Data derived from our in vitro model suggest the use of drug combinations for the treatment of *S. aureus* infections, including those caused by SCVs. Further studies are needed to confirm these suggestions for the treatment of clinical infection.

This work was supported by Cubist Pharmaceuticals and in part by resources and facilities of the Samuel S. Straton VA Medical Center, Albany, NY.

We are grateful to Dianna Bopp, Wadsworth Center, New York State Department of Health, for the performance of the pulsed-field gel electrophoresis DNA fingerprinting of the bacterial isolates. We thank Chendell Sheehan for excellent assistance in the preparation of the manuscript.

### REFERENCES


