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Attenuated Vancomycin Bactericidal Activity against *Staphylococcus aureus hemB* Mutants Expressing the Small-Colony-Variant Phenotype▽

Brian T. Tsuji,[1,2]* Christof von Eiff,[3] Pamela A. Kelchlin,[1] Alan Forrest,[1] and Patrick F. Smith[1,2]†

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, State University of New York, Buffalo, New York 14260[1]; Departments of Pharmacy and Medicine, Roswell Park Cancer Institute, Buffalo, New York 14260[2]; and Institute of Medical Microbiology, University of Münster, Münster, Germany[3]

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The in vitro bactericidal activities of vancomycin against *Staphylococcus aureus hemB* mutants displaying the small-colony-variant phenotype and their parental strains were evaluated. Vancomycin killing activities against *hemB* mutants were markedly attenuated, demonstrating approximately 50% less effect, a result which was well described by a Hill-type pharmacodynamic model.

Staphylococcus aureus has various strategies for resisting therapy that extend beyond classic mechanisms. One of these strategies is the formation of small-colony variants (SCVs), a naturally occurring, slow-growing subpopulation with distinctive phenotypical characteristics and pathogenic traits (12, 13). These fastidious variants characterized by decreased pigment formation, reduced hemolytic activity, and decreased coagulase activity have been associated with chronic, relapsing, and persistent infections (8, 17, 18). The pathogenicity of *S. aureus* SCVs may lie in their ability to persist intracellularly in nonprofessional phagocytes, thus evading surrounding host defenses and antimicrobial agents (4, 11, 23, 26).

In addition to the difficultly of identification in clinical microbiology laboratories, SCVs of *S. aureus* present a potential problem for eradication and microbiologic cure by antimicrobials. Infections caused by methicillin-resistant *S. aureus* (MRSA) SCVs have displayed poor microbiologic responses to certain antimicrobials and are exceptionally difficult to treat (13, 17). Compared to isogenic strains which display the normal phenotype, SCVs have displayed decreased susceptibility to gentamicin, cephalosporins, fluoroquinolones, linezolid, and triclosan (1, 2, 5, 11, 25). There have been no investigations evaluating the pharmacodynamic activity of vancomycin against *S. aureus* SCVs. Therefore, we evaluated the in vitro activities of vancomycin against stable, genetically defined *S. aureus* mutants and a clinical *S. aureus* hemin-auxotrophic strain displaying the SCV phenotype and compared the pharmacodynamics to those for the parental isolates displaying the normal phenotype.

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* Corresponding author. Mailing address: School of Pharmacy and Pharmaceutical Sciences, University at Buffalo, State University of New York, 331 Cooke Hall, Buffalo, NY 14260. Phone: (716) 881-7543. Fax: (716) 645-2886. E-mail: btsuji@buffalo.edu.

† Present address: Roche Pharmaceuticals, Nutley, NJ.

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Bacterial isolates utilized in this study included *S. aureus* strains COL (methicillin resistant) and Newman (methicillin susceptible) and the respective mutants with the SCV phenotype Ia48 (COL *hemB*::*ermB*) and III33 (Newman *hemB*::*ermB*). The construction of both Ia48 and III33 by allelic replacement in *S. aureus* strains COL and Newman has been described previously (7, 22).

Additionally, clinically derived *S. aureus* strains obtained from a patient with a recurrent and persistent infection included A20780 I, displaying the normal phenotype, and the respective hemin-auxotrophic strain displaying the SCV phenotype, A20780 II. A20780 II was recognized as an SCV, clonally identical to the respective normal-phenotype strain, and was genotyped as described previously (23, 24).

Fresh working solutions of vancomycin (analytical-grade powder; Sigma Chemical, St. Louis, MO) were made prior to each experimental run. MICs were determined in quadruplicate using CLSI broth microdilution methods.

Brain heart infusion (BHI) broth (Becton, Dickinson and Company, Franklin Lakes, NJ) supplemented with calcium (25 mg/liter) and magnesium (12.5 mg/liter) was used for all broth microdilution susceptibility testing and time-kill experiments. Briefly, for time-kill experiments, fresh bacterial colonies from an overnight growth were added to normal saline and the turbidity was adjusted to a 0.5 McFarland standard to provide a standard suspension. This suspension was diluted with BHI and a standard antibiotic stock solution to achieve a starting inoculum of approximately 10^7 CFU/ml. Each 10-ml culture was incubated in a water bath at 35°C with constant shaking, and 0.1-ml samples were withdrawn for the determination of bacterial counts at 0, 1, 2, 4, 6, and 24 h. Colony counts were determined by plating 50 μl of each diluted sample onto BHI agar (Becton Dickinson, Franklin Lakes, NJ) with an automated spiral dispenser (WASP; Don Whitley Scientific Limited, West Yorkshire, England) and incubating the plates for 24 h at 35°C to confirm colony counts. Ia48 and III33 were incubated for 48 h. Growth control wells for each organism were prepared without antibiotic and run in parallel with the

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antibiotic test wells. All time-kill experiments were completed in duplicate to quadruplicate.

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 (the 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 ($AUCFU_{0\text{-}24}$) was calculated via the trapezoidal rule for both the growth control ($AUCFU_{growth\ control}$) and the drug-containing regimens ($AUCFU_{drug}$). The $AUCFU_{0\text{-}24}$ was normalized by using the $AUCFU_{0\text{-}24}$ for the growth control, and the logarithm of this ratio was used to quantify the drug effect as shown in equation 1. Additionally, the traditional measure (the log ratio change) for comparing the changes in numbers of CFU per milliliter from 0 (CFU_{0h}) to 24 (CFU_{24h}) hours was calculated as shown in equation 2.

$$\text{Log ratio area} = \log_{10}\left(\frac{AUCFU_{drug}}{AUCFU_{growth\ control}}\right) \quad (1)$$

$$\text{Log ratio change} = \log_{10}\left(\frac{CFU_{24h}}{CFU_{0h}}\right) \quad (2)$$

Using nonlinear regression, a four-parameter concentration-effect Hill-type model was fit to the effect parameter with a software program (version 11) from Systat (Richmond, VA) with the following equation:

$$E = E_0 - \frac{E_{max} \times (C/MIC)^H}{(EC_{50})^H + (C/MIC)^H} \quad (3)$$

The dependent variable (E) is either the log ratio area or the log ratio change, E_0 is the effect measured at a drug concentration of zero, E_{max} is the maximal effect, C is concentration of the drug, C/MIC is the concentration-to-MIC ratio, EC_{50} is the C/MIC ratio for which there is 50% maximal effect, and H is the Hill or sigmoidicity constant.

Vancomycin displayed a MIC for *S. aureus* strains COL, Newman, and A20780 I of 1.0 mg/liter and a MIC for *hemB* mutants Ia48, III33, and A20780 II of 2.0 mg/liter. The killing activities and pharmacodynamics of vancomycin against all isolates are depicted in Fig. 1. With increasing vancomycin concentrations, a concentration-dependent trend toward a greater level of killing of strains COL, Newman, and A20780 I was observed. Vancomycin achieved bactericidal activity against COL and Newman at all concentrations greater than four times the MIC. Killing activity at concentrations above this threshold was sustained until terminal end points at 24 h, with maximal reductions from the baseline of 3.99 and 4.81 $\log_{10}$ CFU/ml at 64 times the MIC for COL and Newman, respectively. Against the clinical isolate A20780 I, vancomycin approached the bactericidal threshold, as the maximal bacterial reduction was 2.39 $\log_{10}$ CFU/ml at 64 times the MIC.

In contrast, against both *hemB* mutants displaying the SCV phenotype, vancomycin activity was markedly attenuated compared to that against the isogenic parental strains with the normal phenotype. Against Ia48 and III33, vancomycin did not achieve bactericidal activity over 24 h, as maximal bacterial reductions from the baseline were 1.40 and 1.88 $\log_{10}$ CFU/ml at 24 h. This trend was also observed against the A20780 II clinical isolate displaying the SCV phenotype, as the maximal bacterial reduction from the baseline was 1.78 CFU/ml at 24 h. Killing activity was less dependent on the concentration for all strains displaying the SCV phenotype, Ia48, III33, and A20780 II, than for strains displaying the normal phenotype, as the activities of vancomycin at 4 to 64 times the MIC for the SCV strains gave similar reductions in bacterial counts at 24 h.

Model-fitted parameter estimates for vancomycin against all strains are displayed in Table 1. An analysis of the 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 over 24 h, revealed better fits to the model, as R^2 was greater than 0.99 for all isolates, and also resulted in lower standard errors for H and E_{max} among most isolates. The bactericidal activity of vancomycin against both those strains displaying the SCV phenotype and those strains displaying the normal phenotype occurred in a concentration-dependent manner. The results from pharmacodynamic comparisons of the strains which displayed the normal phenotype versus the SCVs differed. In particular, the maximal effect of vancomycin was consistently greater for strains which displayed the normal phenotype than for strains which displayed the SCV phenotype.

Vancomycin has been the drug of choice for MRSA infections over the last three decades. However, this role is increasingly challenged by concerns over treatment efficacy. Slow clinical responses, vancomycin's slow bactericidal or bacteriostatic killing activity, and strains which display heterogeneous resistance to vancomycin severely limit its use for persistent infections (6, 9, 15). In the present study, we hypothesized that *hemB* mutants mimicking the SCV phenotype displayed different killing profiles from their isogenic parent strains. Vancomycin displayed reduced killing activities against the mutants, approaching near half the maximal effect, and altered pharmacodynamics compared to those for the parent strains. The findings were consistent when vancomycin was tested against an *S. aureus* SCV clinical strain for comparison to the clonally identical strain displaying the normal phenotype. Of note, the clinical SCV strain displayed some degree of reversion to large colonies on solid medium after 24 h in the absence of vancomycin and at vancomycin concentrations below and above the MIC; however, this reversion did not appear to significantly impact vancomycin pharmacodynamics, as similar trends were observed among stable *hemB* mutants.

The reduced activity of vancomycin against SCVs may be due in part to the expression patterns of global regulators σ^B and the accessory gene regulator (*agr*) in SCVs of *S. aureus*. SCVs of *S. aureus* isolated from cystic fibrosis patients and *hemB* mutants have been shown previously to be influenced by σ^B, a stress-induced transcription factor. Interestingly, it has been demonstrated previously that σ^B reduces the levels of RNA III, the effector molecule of the *agr* system, in a growth phase-dependent manner (3, 20). Additionally, other investigations have shown that SCVs consistently display low membrane potentials, reduced intracellular ATP concentrations, and reduced RNA III production, resulting in reduced cell wall biosynthesis (10, 16, 19). Furthermore, we tested both *hemB* SCV mutants for delta-hemolysin expression, a marker for the *agr* function, and both SCV isolates were delta-hemolysin negative (14, 21). Taken together with our recent findings that a

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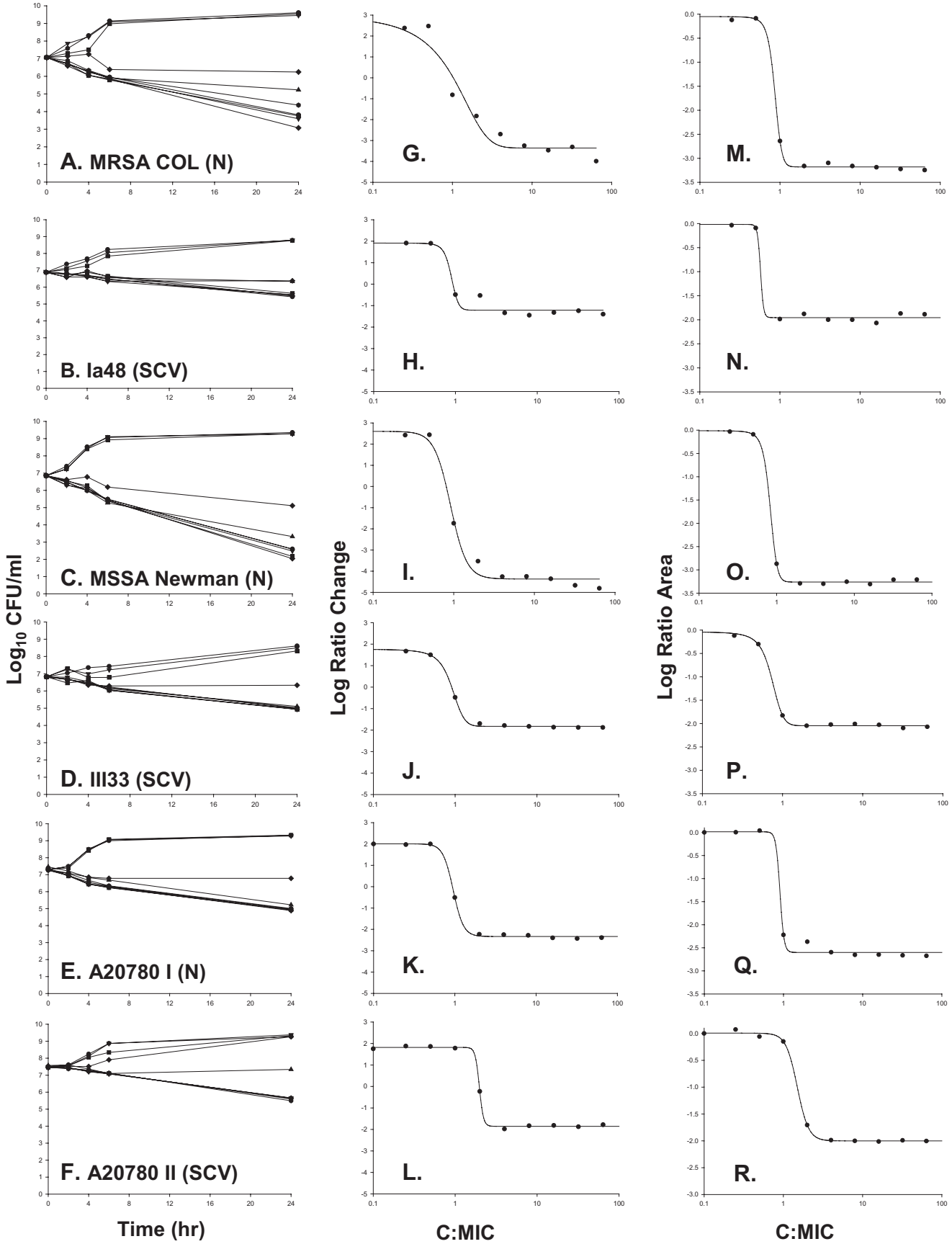

FIG. 1. Results from vancomycin time-kill experiments evaluating bactericidal activity toward *S. aureus* strains displaying the normal (N) and SCV phenotypes. The key to symbols for each regimen is as follows: control (●) and 0.25 (▼), 0.5 (■), 1 (◆), 2 (▲), 4 (●), 8 (●), 16 (▼), 32 (■), and 64 (◆) times the MIC. MSSA, methicillin-susceptible *S. aureus*.

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TABLE 1. Model-fitted parameter estimates[a] for vancomycin versus *S. aureus* strains displaying the normal and SCV phenotypes

Parameter	Value for *S. aureus* strain:					
	COL	Ia48	Newman	III33	A20780 I	A20780 II
Log ratio area parameters						
E_0	−0.060 (70.0)	0.018	−0.016 (>100)	−0.057 (57.8)	0.014 (>100)	0.00 (>100)
E_{max}	3.12 (1.57)	1.94	3.25 (1.05)	1.99 (1.91)	2.61 (3.36)	2.00 (1.40)
H	8.97 (39.8)	15.0[b]	8.29 (12.29)	5.82 (8.52)	17.2 (>100)	6.08 (7.91)
EC_{50}	0.840 (7.14)	0.618	0.787 (3.12)	0.699 (3.14)	0.902 (>100)	1.50 (>100)
R^2	0.999	0.995	0.999	0.998	0.999	0.999
Log ratio change parameters						
E_0	2.77 (15.87)	1.93 (11.5)	2.60 (10.5)	1.75 (1.95)	2.00 (2.62)	1.81 (2.35)
E_{max}	6.21 (8.83)	3.14 (8.23)	6.97 (4.73)	3.59 (1.14)	4.34 (1.49)	3.67 (1.60)
H	2.11 (28.91)	6.16 (85.9)	4.33 (30.45)	4.29 (7.05)	6.89 (34.4)	17.56 (>100)
EC_{50}	1.07 (16.1)	0.839 (16.9)	0.991 (5.65)	0.895 (1.56)	0.955 (2.02)	1.97 (6.59)
R^2	0.974	0.971	0.991	0.999	0.999	0.995

[a] Data are reported as maximum-likelihood parameter estimates (percent standard errors are shown in parentheses). E_0, effect at concentration-to-MIC ratio of 0; E_{max}, maximal effect (values expressed as $\log_{10}$ numbers of CFU per milliliter); H, Hill's constant; EC_{50}, concentration-to-MIC ratio for which there is 50% maximal effect.

[b] Hill's constant was fixed at 15.0, and the percent standard error is not shown.

down-regulated *agr* locus is associated with the development of vancomycin intermediate resistance at therapeutically relevant concentrations, this outcome may provide further explanation of our results which demonstrate attenuated vancomycin activity against *hemB* mutants (14, 21). Additionally, the lack of bactericidal activity over a wide range of concentrations and the altered pharmacodynamics among *hemB* mutants also highlight the lack of utility of the MIC alone to predict vancomycin efficacy. Coupled with the suboptimal penetration of vancomycin into some sites of infection, such as epithelial lining fluid in cases of MRSA pneumonia, and the ability of SCVs to persist intracellularly, these findings highlight the difficulty in eradicating *S. aureus* SCVs in serious, deep-seated infections by glycopeptide monotherapy. These findings also suggest that administering significantly higher doses of vancomycin to combat *S. aureus* SCV infections may not be beneficial, especially in the light of increased concerns for nephrotoxicity. With recent reports that suggest that SCVs may represent a natural component of the life cycle of staphylococci (13), our findings are of particular interest and may have implications for the optimal treatment of *S. aureus* infections.

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