AAC Journals.ASM.org

Evaluation of Ceftaroline Activity against Heteroresistant Vancomycin-Intermediate *Staphylococcus aureus* and Vancomycin-Intermediate Methicillin-Resistant *S. aureus* Strains in an *In Vitro* Pharmacokinetic/Pharmacodynamic Model: Exploring the "Seesaw Effect"

Brian J. Werth,[a] Molly E. Steed,[a] Glenn W. Kaatz,[b,c,d] Michael J. Rybak[a,b,c]

Anti-Infective Research Laboratory, Department of Pharmacy Practice, Eugene Applebaum College of Pharmacy and Health Sciences[a] and Department of Internal Medicine, Division of Infectious Diseases,[b] School of Medicine, Wayne State University,[c] Detroit, Michigan, USA; John D. Dingell Veterans Affairs Medical Center, Detroit, Michigan, USA[d]

A "seesaw effect" in methicillin-resistant *Staphylococcus aureus* (MRSA) has been demonstrated, whereby susceptibility to β-lactam antimicrobials increases as glyco- and lipopeptide susceptibility decreases. We investigated this effect by evaluating the activity of the anti-MRSA cephalosporin ceftaroline against isogenic pairs of MRSA strains with various susceptibilities to vancomycin in an *in vitro* pharmacokinetic/pharmacodynamic (PK/PD) model. The activities of ceftaroline at 600 mg every 12 h (q12h) (targeted free maximum concentration of drug in serum [fC_{max}], 15.2 μg/ml; half-life [$t_{1/2}$], 2.3 h) and vancomycin at 1 g q12h (targeted fC_{max}, 18 μg/ml; $t_{1/2}$, 6 h) were evaluated against 3 pairs of isogenic clinical strains of MRSA that developed increased MICs to vancomycin in patients while on therapy using a two-compartment hollow-fiber PK/PD model with a starting inoculum of ~10^7 CFU/ml over a 96-h period. Bacterial killing and development of resistance were evaluated. Expression of penicillin-binding proteins (PBPs) 2 and 4 was evaluated by reverse transcription (RT)-PCR. The achieved pharmacokinetic parameters were 98 to 119% of the targeted values. Ceftaroline and vancomycin were bactericidal against 5/6 and 1/6 strains, respectively, at 96 h. Ceftaroline was more active against the mutant strains than the parent strains, with this difference being statistically significant for 2/3 strain pairs at 96 h. The level of PBP2 expression was 4.4× higher in the vancomycin-intermediate *S. aureus* (VISA) strain in 1/3 pairs. The levels of PBP2 and PBP4 expression were otherwise similar between the parent and mutant strains. These data support the seesaw hypothesis that ceftaroline, like traditional β-lactams, is more active against strains that are less susceptible to vancomycin even when the ceftaroline MICs are identical. Further research to explore these unique findings is warranted.

Ceftaroline (CPT) is an anti-methicillin-resistant *Staphylococcus aureus* (anti-MRSA) cephalosporin approved by the U.S. Food and Drug Administration as the prodrug ceftaroline-fosamil (CPT-F) for the treatment of community-acquired bacterial pneumonia and acute bacterial skin and skin structure infections. CPT inhibits cell wall synthesis by irreversibly binding penicillin-binding proteins (PBPs) 1 to 3, including the mutated PBP2a, which confers methicillin resistance, but like most β-lactams, CPT has minimal affinity for PBP4 (1–4). CPT maintains activity against MRSA isolates with reduced susceptibility to vancomycin (VAN) and daptomycin (DAP), including heteroresistant VAN-intermediate *S. aureus* (hVISA), VISA, VAN-resistant *S. aureus* (VRSA), and DAP-nonsusceptible *S. aureus* (DNSSA) (5, 6). Experiments evaluating CPT activity in a previously described *in vitro* pharmacokinetic/pharmacodynamic (PK/PD) hollow fiber model against MRSA isolates with reduced susceptibility to VAN or DAP have suggested CPT may exhibit enhanced activity against hVISA and VISA compared to that in VAN-susceptible *S. aureus* (VSSA) (7, 8). Although this observation was unrelated to the objectives of these studies, it was noted with all of the hVISA and VISA strains, with the exception of Mu3, which is not susceptible to CPT.

The previously described "seesaw" effect in MRSA, in which isolates show increased oxacillin (OXA) susceptibility as VAN susceptibility decreases, may contribute to the enhanced bactericidal activity of CPT against hVISA and VISA isolates (9, 10). While the exact mechanism for increases in OXA susceptibility in the presence of the *mecA* gene is not fully elucidated, genetic and/or metabolic modifications in the expression or nature of PBPs due to VAN or DAP pressure may contribute (11). Comparisons of both unrelated clinical strains and *in vitro*-derived pairs have revealed a decrease or absence of PBP4 in VISA strains compared to VAN-susceptible MRSA (11–13). Studies examining both *in vitro* and clinical pairs have found increases in either the amount of PBP2, levels of expression of PBPs, or degree of PBP2 activity in VISA or VISA-like strains compared to those in VAN-susceptible *S. aureus* strains (13–15). A decrease in PBP4, to which CPT has minimal affinity, coupled with the increase in PBP2, to which CPT has high affinity, may explain the enhanced activity of β-lactams, including CPT, in hVISA and VISA strains.

The objective of the present study was to investigate the poten-

Received 20 November 2012 **Returned for modification** 21 December 2012
Accepted 15 March 2013
Published ahead of print 1 April 2013
Address correspondence to Michael J. Rybak, m.rybak@wayne.edu.

doi:10.1128/AAC.02308-12

TABLE 1 Vancomycin phenotypes, susceptibilities, and population analyses of the strains tested in this study

Strain	Phenotype	MIC (μg/ml) by BMD (MIC by Etest)			VAN PAP AUC ratio[a]	CPT PAP AUC
		VAN	CPT	DAP		
JH1	VSSA	1 (2)	0.5 (0.5)	0.25 (0.38)	0.8247	7.95
JH9	VISA	8 (8)	0.5 (0.5)	1 (1.5)	NP	7.45
R6911	hVISA	2 (4)	0.5 (0.5)	2 (1.5)	1.2528	10.13
R6913	VISA	4 (8)	0.5 (0.5)	4 (8)	NP	8.99
T51643	VSSA	1 (2)	1 (1)	0.5 (0.5)	0.774	12.21
H9749-2	hVISA	2 (3)	1 (0.5)	0.5 (1.5)	1.167	10.32
Mu3	hVISA	2 (3)	2 (1.5)		1	13.42
Mu50	hVISA	4 (4)	1 (0.75)		NP	8.16

[a] The AUC ratio is relative to the PAP AUC of Mu3. A VAN PAP AUC ratio of ≥0.9 is considered positive for the hVISA phenotype. Vancomycin population analysis was not performed (NP) on known VISA strains.

tial enhanced activity of CPT against MRSA strains with reduced susceptibility to VAN and/or DAP. This was accomplished by comparing the difference in killing by CPT in isogenic strain pairs that vary only in their susceptibility to VAN and DAP in a two-compartment hollow fiber PK/PD model and by quantifying changes in PBP2 and PBP4 expression between these strains.

MATERIALS AND METHODS

Bacterial strains. Three isogenic clinical MRSA strain pairs, which developed reduced susceptibility to VAN *in vivo* in patients on therapy with VAN or DAP, were evaluated. The hVISA strain Mu3 was used as a reference strain for population analysis experiments as previously described (16). The VISA strain Mu50 was also included in the CPT population analysis experiments as a comparator. The strain information is summarized in Table 1.

Antimicrobials and media. CPT was provided by its manufacturer (Forest Laboratories, Inc., New York, NY). VAN and DAP were commercially purchased (Sigma-Aldrich Co., St. Louis, MO, and Cubist Pharmaceuticals, Lexington, MA, respectively). Mueller-Hinton broth (MHB; Difco, Detroit, MI) with 25 mg/liter calcium and 12.5 mg/liter magnesium was used for all *in vitro* experiments. MHB supplemented to 50 mg/liter of calcium was used for DAP MIC testing. Colony counts were determined using tryptic soy agar (TSA; Difco) plates. Brain heart infusion agar (BHIA; Difco) plates, supplemented with VAN or CPT, were used for resistance screening and population analysis experiments. Antibiotic medium agar 11 (Difco, Detroit, MI) was used for bioassays performed for pharmacokinetic analysis.

Susceptibility testing. The MICs of study antimicrobial agents were determined by broth microdilution (BMD) according to Clinical and Laboratory Standards Institute (CLSI) guidelines and by Etest (17). All samples were incubated at 35°C for 24 h before the MICs were read.

Modified PAP. A bacterial suspension of 1×10^8 CFU/ml was plated with an automatic spiral-plating device (WASP; DW Scientific, West Yorkshire, United Kingdom) onto freshly prepared BHIA plates containing 0.06 to 2.5 μg/ml of CPT or 0.25 to 8 μg/ml of VAN (7, 16). After 48 h of incubation at 35°C, colony counts were determined using an automated colony counter (ProtoCOL; Synoptics, Ltd., Frederick, MD). The lower limit of detection for colony counts is 2 $\log_{10}$ CFU/ml. Colony counts were plotted against increasing concentrations of VAN or CPT, and the areas under the curve (AUC) of the resultant curves were calculated using SigmaPlot (version 10.0; Systat Software, Inc., San Jose, CA). For VAN population analysis profiles (PAPs), AUC ratios were calculated by dividing the AUC of the test organism by the AUC of hVISA strain Mu3, which was determined on the same day. Any strain with an AUC ratio of ≥0.9 was considered positive for the hVISA phenotype (16).

***In vitro* PK/PD model.** An *in vitro* two-compartment hollow fiber PK/PD model (model C2011; Fiber Cell Systems, Inc., Frederick, MD) was utilized, inoculated, and maintained as previously described (7). Fresh medium was continuously supplied and removed from the central compartment along with the drug via a peristaltic pump (Masterflex; Cole-Parmer Instrument Company, Chicago, IL) set to simulate the average human half-lives ($t_{1/2}$) of the antibiotics. The antibiotic regimens were free drug simulations of CPT-F at 600 mg every 12 h (q12h) ($t_{1/2}$, 2.3 h; targeted free maximum concentration of drug in serum [fC_{max}], 15.2 μg/ml; protein binding, 20%), and VAN at 1,000 mg q12h ($t_{1/2}$, 6 h; targeted fC_{max}, 18 μg/ml; protein binding, 50%). A drug-free growth control was also run for each strain. All model tests were performed in duplicate to ensure reproducibility.

Pharmacodynamic analysis. Samples from each model were collected at 0, 4, 8, 24, 28, 32, 48, 56, 72, and 96 h in duplicate and diluted in cold 0.9% saline. Colony counts were performed as previously described (7). Changes in $\log_{10}$ CFU/ml were plotted against time to construct curves to describe the antibacterial activities of the simulated regimens. Bactericidal activity (99.9% kill) was defined as a ≥3-$\log_{10}$-CFU/ml decrease in colony count from the initial inoculum. Bacteriostatic activity was defined as a <3-$\log_{10}$-CFU/ml reduction in colony count from the initial inoculum, while inactivity was defined as no observed reduction in initial inoculum.

Pharmacokinetic analysis. Duplicate pharmacokinetic samples were obtained and stored as previously described (7). CPT and VAN concentrations were determined by bioassay and fluorescence polarization immunoassay (TDX assay; Abbott Diagnostics), respectively, using previously described methods (7, 18). The intraday coefficients of variation for the VAN and CPT assays are <2.5% and <10%, respectively, for high, medium, and low standards. The half-lives, area under the curve (AUC), and peak concentrations of the antibiotics were determined by the trapezoidal method utilizing PK Analyst software (version 1.10; MicroMath Scientific Software, Salt Lake City, UT).

Resistance. Emergence of resistance was evaluated at multiple time points throughout the simulation by plating 100-μl samples from each time point on BHIA plates supplemented with CPT or VAN at a concentration 3× the MIC of the tested antibiotic. Plates were examined for growth after 24 and 48 h of incubation at 35°C.

Determination of PBP expression levels in isogenic organism pairs. Expression levels of PBP2 and PBP4 were determined by quantitative RT-PCR in the strains with reduced susceptibility to VAN and compared to those of the isogenic parent strain by previously described methods (19). Briefly, each strain was grown overnight in MHB supplemented with 0.5× the MIC of VAN. Cells from overnight cultures were used to inoculate 50 ml of Mueller-Hinton broth, and the cells were allowed to grow to an optical density at 550 nm (OD_{550}) of 0.4 and harvested by centrifugation. Total RNA was isolated from cell pellets of the isogenic strain pairs and immediately incubated with 1 ml RNAprotect (Qiagen, Valencia, CA). Total RNA was isolated using a Purescript RNA isolation kit (Gentra, Minneapolis, MN) according to the manufacturer's instructions. Quantitative RT-PCR was performed using a one-step RT-PCR kit (Qiagen) following the manufacturer's recommended protocol. TaqMan probes and primers for PBP2 and PBP4 were designed using Beacon Designer 7.80 (Premier Biosoft International, Palo Alto, CA) and were purchased commercially (Eurofins MWG/Operon, Huntsville, AL). 16S RNA was used as a housekeeping gene control. Each reaction was performed in triplicate. PBP transcription was considered to be induced or downregulated when mRNA was present at a level at least 4-fold higher or lower than that of the corresponding MRSA parent strain (19).

Statistical analysis. Changes in $\log_{10}$ CFU/ml at 24, 48, 56, 72, and 96 h were compared by one-way analysis of variance (ANOVA). A P value of ≤0.05 was considered significant. All statistical analyses were performed using SPSS statistical software (release 20.0; SPSS, Inc., Chicago, IL).

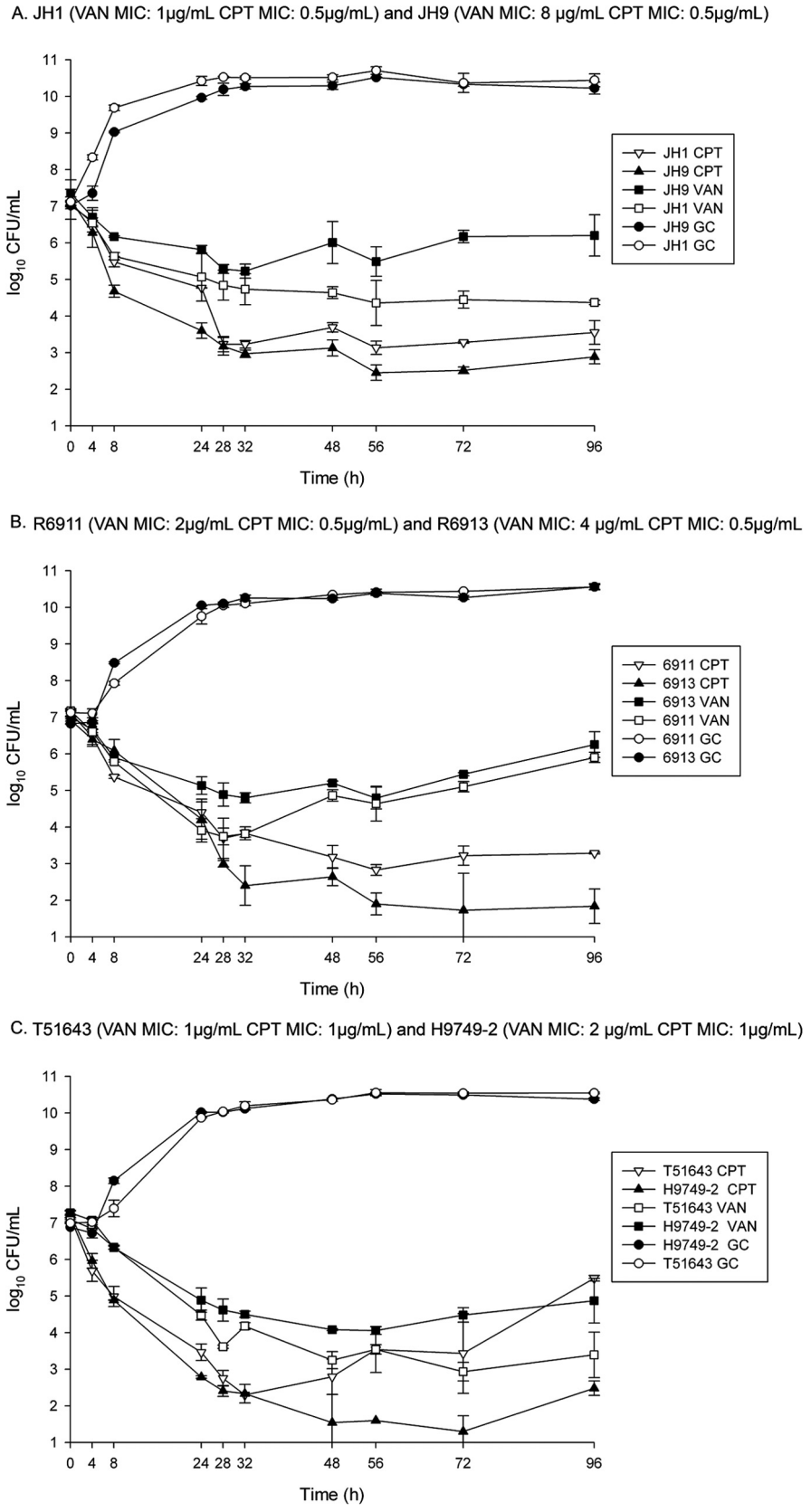

FIG 1 Activity of simulated drug regimens tested against each isogenic strain pair in the *in vitro* hollow fiber PK/PD model.

RESULTS

Susceptibility and population analysis data for each pair are summarized in Table 1. The CPT MICs were 0.5 or 1 μg/ml, with no differences between the parent and mutant strains for any of the three isogenic pairs by standard BMD methods. VAN MICs were 1 or 2 μg/ml in parents and increased to 2 to 8 μg/ml in VISA and/or hVISA derivatives by standard BMD methods. DAP MICs were 0.25/1 μg/ml, 2/4 μg/ml, and 0.5/0.5 μg/ml for the parent/derivative isogenic strain pairs, respectively, by standard BMD methods. Etest MICs were higher than the BMD values for VAN but were similar for CPT and DAP.

The average observed fC_{max} values for VAN and CPT were 21.5 ± 0.2 μg/ml (target, 18 μg/ml) and 14.63 ± 0.3 μg/ml (target, 15.2 μg/ml), respectively. The average observed half-lives for VAN and CPT were 5.4 ± 0.16 h (target, 6 h) and 2.27 ± 0.26 h (target, 2.3 h), respectively. The VAN $fAUC_{0-24}$ was 218 ± 3.25 mg · h/ml. For CPT, the free-drug times above the MIC were 91.35% of the dosing interval for the strains with a MIC of 0.5 μg/ml and 72.5% for the strains with a MIC of 1 μg/ml.

Bacterial survival against each antimicrobial regimen over time for each strain pair is summarized in Fig. 1A to C. CPT was significantly more active against the mutant strain than the parent strain by 96 h in 2/3 pairs and maintained bactericidal activity against 5/6 strains by 96 h. VAN was bacteriostatic against 5/6 strains by 96 h. VAN, at a simulated dose of 1 g intravenous (i.v.) every 12 h, was less active than CPT in this model against all strains except for one VAN-susceptible MRSA strain (T-51643). VAN was significantly more active against JH1 (MIC, 1 μg/ml) than its VISA mutant, JH9 (MIC, 8 μg/ml), and was somewhat more active against T-51643 than its hVISA derivative, H9749-2, but was similarly active against the hVISA-VISA pair R6911 and R6913. The emergence of resistance was not detected from any of the models.

VAN PAP was conducted on non-VISA strains in order to identify hVISA. R6911 and H9749-2 were both positive for the hVISA phenotype, as indicated by a PAP AUC ratio to hVISA control strain Mu3 of ≥0.9 (Table 1) (16). CPT population analysis was also performed in order to describe heterogeneous susceptibility to CPT (Fig. 2). Values representing heterogeneity of susceptibility to CPT and VAN, reported as PAP AUC or AUC ratios to Mu3, are listed in Table 1. The CPT AUC values were higher in the more VAN-susceptible than in the less-susceptible mutant strain in each pair. A larger difference in CPT AUC between parent and mutant strains seemed to correlate with a larger difference in CPT killing in the model; however, there was poor correlation between CPT AUC and killing when all strains were compared in all models.

PBP4 was expressed to a lesser extent in the strain with reduced susceptibility to VAN in each pair; however, this difference was too small (<4-fold) to be considered a substantial change in expression. JH1 expressed 1.3 times more PBP4 than JH9, R6911 expressed 1.1 times more PBP4 than R6913, and T51643 expressed 2 times more PBP4 than H9749-2. PB2 expression was substantially induced in JH9 relative to that in JH1 but was moderately downregulated in the mutant strains of the other pairs. PBP2 was expressed 4.4 times higher in JH9 than in JH1, but expression was 63% lower in R6913 than that in R6911 and 28% lower in H9749-2 than that in T51643.

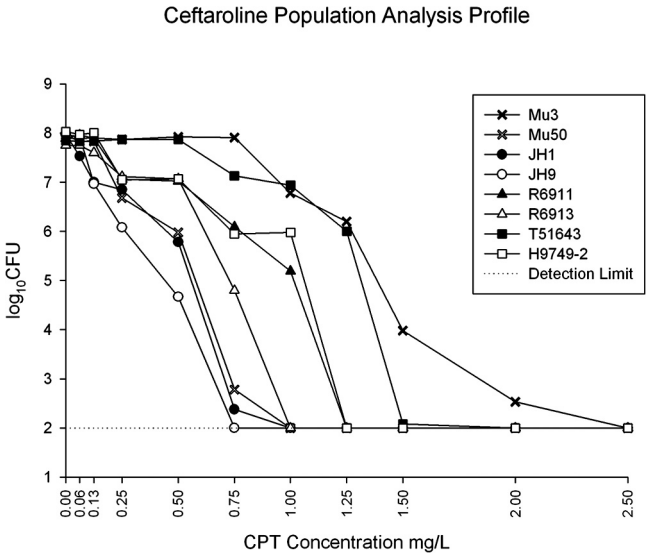

FIG 2 Ceftaroline population analysis profile for various strains tested in this study. Shown are the ceftaroline population analysis profiles for Mu3 (hVISA; VAN MIC, 2 μg/ml; CPT MIC, 2 μg/ml), JH1 (VSSA; VAN MIC, 1 μg/ml; CPT MIC, 0.5 μg/ml), JH9 (VISA; VAN MIC, 8 μg/ml; CPT MIC, 0.5 μg/ml), R6911 (hVISA; VAN MIC, 2 μg/ml; CPT MIC, 0.5 μg/ml), R6913 (VISA; VAN MIC, 4 μg/ml; CPT MIC, 0.5 μg/ml), T51643 (VSSA; VAN MIC, 1 μg/ml; CPT MIC, 1 μg/ml), and H9749-2 (hVISA; VAN MIC, 2 μg/ml; CPT MIC, 1 μg/ml).

DISCUSSION

The inverse correlation between β-lactam susceptibility and lipo- and glycopeptide susceptibility in MRSA, known as the seesaw effect, has been shown to affect traditional antistaphylococcal β-lactams. However, it is unknown whether this relationship holds true for the anti-MRSA cephalosporin CPT. In this study, we evaluated differences in CPT activity in closely related isolates that differed only in the susceptibilities to VAN and DAP. Interestingly, in all three pairs the mutant and the parent strain had the same CPT MIC. We demonstrated that CPT was significantly more active against MRSA strains with reduced susceptibility to VAN relative to their more susceptible parent strains for 2/3 pairs tested, despite the fact that both parent and mutant strains had the same CPT MIC. This observation supports the hypothesis that CPT is also affected by the seesaw effect and may be more active against strains with a higher VAN and/or DAP MIC. Even though there was not a measurable difference in the CPT MIC for the mutant versus the parent strain in any of the pairs, using a population analysis profile to characterize less-susceptible subpopulations we revealed that mutant strains were more uniformly susceptible than their corresponding parent strains, as evidenced by their lower CPT PAP AUC. The relative decrease in heterogeneity between the parent and mutant strains seemed to be proportional to the increased level of killing observed in the model. However, there was a poor correlation between the $\log_{10}$-CFU/ml reduction in bacterial load and the CPT PAP AUC across all strains (data not shown). The simulated CPT regimen resulted in greater bacterial killing than the simulated VAN regimen against all strains, except for T51643 (MRSA). It is interesting to note that this strain was the most susceptible to VAN (VAN MIC, 1 μg/ml; VAN PAP AUC ratio, 0.77) and the least susceptible to CPT (CPT MIC, 1 μg/ml; CPT PAP AUC, 12.21). The improved activity of CPT simulations

relative to VAN was not surprising, as the VAN exposures in the model were only optimized for the two VSSA strains with MICs of 1 μg/ml. Suboptimal VAN exposure in this model does not allow for clinical extrapolation of the comparison between CPT and VAN; however, this was not the goal of this experiment.

The improvement in CPT killing against less-susceptible strains was not associated with a substantial reduction in transcription of PBP4, as was hypothesized. The expected increase in PBP2 expression in VAN nonsusceptible strains was only observed between JH1 and JH9. Other investigators have shown that PBP2 and PBP2a are both induced in the presence of cell wall active agents, including VAN and OXA (15, 20). While some have suggested that reduction of PBP4 transcription plays an important role in the expression of the VISA phenotype, others have found that PBP4, as well as other PBP subtypes, may be increased or unchanged in glycopeptide-nonsusceptible strains (11, 12, 21). It is possible that differential PBP expression is a common but not universal mechanism for reduced VAN susceptibility and the see-saw effect. Further research is warranted to clarify the role of variable PBP expression in glycopeptide-nonsusceptible strains.

ACKNOWLEDGMENTS

This work was funded by an investigator-initiated grant from Forest Laboratories. M.J.R. is funded in part by NIH R21A1092055-01.

We thank Abbott Laboratories for the use of the fluorescence polarization immunoassay analyzer for determination of vancomycin concentrations. We also thank Alexander Tomasz (The Rockefeller University, New York, NY) for providing strains JH-1 and JH-9.

M.J.R. has received grant support, consulted for, or provided lectures for Astellas, Cubist, Forest, Pfizer, Novartis, and Rib-X. B.J.W., M.E.S., and G.W.K. have no potential conflicts of interest to declare.

REFERENCES

1. **Villegas-Estrada A, Lee M, Hesek D, Vakulenko SB, Mobashery S.** 2008. Co-opting the cell wall in fighting methicillin-resistant Staphylococcus aureus: potent inhibition of PBP 2a by two anti-MRSA beta-lactam antibiotics. J. Am. Chem. Soc. **130**:9212–9213.
2. **Steed ME, Rybak MJ.** 2010. Ceftaroline: a new cephalosporin with activity against resistant gram-positive pathogens. Pharmacotherapy **30**:375–389.
3. **Kosowska-Shick K, McGhee PL, Appelbaum PC.** 2010. Affinity of ceftaroline and other beta-lactams for penicillin-binding proteins from Staphylococcus aureus and Streptococcus pneumoniae. Antimicrob. Agents Chemother. **54**:1670–1677.
4. **Moisan H, Pruneau M, Malouin F.** 2010. Binding of ceftaroline to penicillin-binding proteins of Staphylococcus aureus and Streptococcus pneumoniae. J. Antimicrob. Chemother. **65**:713–716.
5. **Sader HS, Fritsche TR, Kaniga K, Ge Y, Jones RN.** 2005. Antimicrobial activity and spectrum of PPI-0903M (T-91825), a novel cephalosporin, tested against a worldwide collection of clinical strains. Antimicrob. Agents Chemother. **49**:3501–3512.
6. **Saravolatz L, Pawlak J, Johnson L.** 2010. In vitro activity of ceftaroline against community-associated methicillin-resistant, vancomycin-intermediate, vancomycin-resistant, and daptomycin-nonsusceptible Staphylococcus aureus isolates. Antimicrob. Agents Chemother. **54**:3027–3030.
7. **Vidaillac C, Leonard SN, Rybak MJ.** 2009. In vitro activity of ceftaroline against methicillin-resistant Staphylococcus aureus and heterogeneous vancomycin-intermediate S. aureus in a hollow fiber model. Antimicrob. Agents Chemother. **53**:4712–4717.
8. **Steed M, Vidaillac C, Rybak MJ.** 2011. Evaluation of ceftaroline activity versus daptomycin (DAP) against DAP-nonsusceptible methicillin-resistant Staphylococcus aureus strains in an in vitro pharmacokinetic/pharmacodynamic model. Antimicrob. Agents Chemother. **55**:3522–3526.
9. **Dhand A, Bayer AS, Pogliano J, Yang SJ, Bolaris M, Nizet V, Wang G, Sakoulas G.** 2011. Use of antistaphylococcal β-lactams to increase daptomycin activity in eradicating persistent bacteremia due to methicillin-resistant Staphylococcus aureus: role of enhanced daptomycin binding. Clin. Infect. Dis. **53**:158–163.
10. **Mwangi MM, Wu SW, Zhou Y, Sieradzki K, de Lencastre H, Richardson P, Bruce D, Rubin E, Myers E, Siggia ED, Tomasz A.** 2007. Tracking the in vivo evolution of multidrug resistance in Staphylococcus aureus by whole-genome sequencing. Proc. Natl. Acad. Sci. U. S. A. **104**:9451–9456.
11. **Finan JE, Archer GL, Pucci MJ, Climo MW.** 2001. Role of penicillin-binding protein 4 in expression of vancomycin resistance among clinical isolates of oxacillin-resistant Staphylococcus aureus. Antimicrob. Agents Chemother. **45**:3070–3075.
12. **Sieradzki K, Pinho MG, Tomasz A.** 1999. Inactivated pbp4 in highly glycopeptide-resistant laboratory mutants of Staphylococcus aureus. J. Biol. Chem. **274**:18942–18946.
13. **Sieradzki K, Tomasz A.** 1999. Gradual alterations in cell wall structure and metabolism in vancomycin-resistant mutants of Staphylococcus aureus. J. Bacteriol. **181**:7566–7570.
14. **Pieper R, Gatlin-Bunai CL, Mongodin EF, Parmar PP, Huang ST, Clark DJ, Fleischmann RD, Gill SR, Peterson SN.** 2006. Comparative proteomic analysis of Staphylococcus aureus strains with differences in resistance to the cell wall-targeting antibiotic vancomycin. Proteomics **6**:4246–4258.
15. **Boyle-Vavra S, Yin S, Challapalli M, Daum RS.** 2003. Transcriptional induction of the penicillin-binding protein 2 gene in Staphylococcus aureus by cell wall-active antibiotics oxacillin and vancomycin. Antimicrob. Agents Chemother. **47**:1028–1036.
16. **Wootton M, Howe RA, Hillman R, Walsh TR, Bennett PM, MacGowan AP.** 2001. A modified population analysis profile (PAP) method to detect hetero-resistance to vancomycin in Staphylococcus aureus in a UK hospital. J. Antimicrob. Chemother. **47**:399–403.
17. **Clinical and Laboratory Standards Institute.** 2012. Performance standards for antimicrobial susceptibility testing: 22nd informational supplement. Clinical and Laboratory Standards Institute, Wayne, PA.
18. **Werth BJ, Sakoulas G, Rose WE, Pogliano J, Tewhey R, Rybak MJ.** 2013. Ceftaroline increases membrane binding and enhances the activity of daptomycin against daptomycin-nonsusceptible vancomycin-intermediate Staphylococcus aureus in a pharmacokinetic/pharmacodynamic model. Antimicrob. Agents Chemother. **57**:66–73.
19. **DeMarco CE, Cushing LA, Frempong-Manso E, Seo SM, Jaravaza TA, Kaatz GW.** 2007. Efflux-related resistance to norfloxacin, dyes, and biocides in bloodstream isolates of Staphylococcus aureus. Antimicrob. Agents Chemother. **51**:3235–3239.
20. **Shlaes DM, Shlaes JH, Vincent S, Etter L, Fey PD, Goering RV.** 1993. Teicoplanin-resistant Staphylococcus aureus expresses a novel membrane protein and increases expression of penicillin-binding protein 2 complex. Antimicrob. Agents Chemother. **37**:2432–2437.
21. **Moreira B, Boyle-Vavra S, deJonge BL, Daum RS.** 1997. Increased production of penicillin-binding protein 2, increased detection of other penicillin-binding proteins, and decreased coagulase activity associated with glycopeptide resistance in Staphylococcus aureus. Antimicrob. Agents Chemother. **41**:1788–1793.