

Evaluation of the Novel Combination of High-Dose Daptomycin plus Trimethoprim-Sulfamethoxazole against Daptomycin-Nonsusceptible Methicillin-Resistant *Staphylococcus aureus* Using an *In Vitro* Pharmacokinetic/Pharmacodynamic Model of Simulated Endocardial Vegetations

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Daptomycin-nonsusceptible (DNS) *Staphylococcus aureus* is found in difficult-to-treat infections, and the optimal therapy is unknown. We investigated the activity of high-dose (HD) daptomycin plus trimethoprim-sulfamethoxazole de-escalated to HD daptomycin or trimethoprim-sulfamethoxazole against 4 clinical DNS methicillin-resistant *S. aureus* (MRSA) isolates in an *in vitro* pharmacokinetic/pharmacodynamic model of simulated endocardial vegetations (10^9 CFU/g). Simulated regimens included HD daptomycin at 10 mg/kg/day for 14 days, trimethoprim-sulfamethoxazole at 160/800 mg every 12 h for 14 days, HD daptomycin plus trimethoprim-sulfamethoxazole for 14 days, and the combination for 7 days de-escalated to HD daptomycin for 7 days and de-escalated to trimethoprim-sulfamethoxazole for 7 days. Differences in CFU/g (at 168 and 336 h) were evaluated by analysis of variance (ANOVA) with a Tukey's *post hoc* test. Daptomycin MICs were 4 μ g/ml (SA H9749-1, vancomycin-intermediate *Staphylococcus aureus*; R6212, heteroresistant vancomycin-intermediate *Staphylococcus aureus*) and 2 μ g/ml (R5599 and R5563). Trimethoprim-sulfamethoxazole MICs were $\leq 0.06/1.19$ μ g/ml. HD daptomycin plus trimethoprim-sulfamethoxazole displayed rapid bactericidal activity against SA H9749-1 (at 7 h) and R6212 (at 6 h) and bactericidal activity against R5599 (at 72 h) and R5563 (at 36 h). A $\geq 8 \log_{10}$ CFU/g decrease was observed with HD daptomycin plus trimethoprim-sulfamethoxazole against all strains (at 48 to 144 h), which was maintained with de-escalation to HD daptomycin or trimethoprim-sulfamethoxazole at 336 h. The combination for 14 days and the combination for 7 days de-escalated to HD daptomycin or trimethoprim-sulfamethoxazole was significantly better than daptomycin monotherapy ($P < 0.05$) and trimethoprim-sulfamethoxazole monotherapy ($P < 0.05$) at 168 and 336 h. Combination therapy followed by de-escalation offers a novel bactericidal therapeutic alternative for high-inoculum, serious DNS MRSA infections.

Daptomycin is a concentration-dependent cyclic lipopeptide antibiotic with activity against Gram-positive organisms that exerts its bactericidal activity by binding to and inserting into bacterial membranes, thereby leading to rapid membrane depolarization and deregulation of several cell functions, such as DNA, RNA, and protein synthesis (9, 18, 31). Daptomycin susceptibility in *Staphylococcus aureus* is defined as a MIC of ≤ 1 μ g/ml, and any strains with a MIC of > 1 μ g/ml are referred to as daptomycin nonsusceptible (DNS) (8). The rate of DNS *S. aureus* in North America over the last several years is low (0.01 to 0.1%), with no trend in increasing MIC values (7, 25, 29, 30). The development of DNS in *S. aureus* is due to a series of incremental changes in the cell membrane and wall due to changes in gene expression or the development of genetic mutations that increase the MIC and eventually lead to DNS strains (13–16, 23, 27, 33, 37). This variability in genetic alterations leading to DNS may explain why daptomycin retains some activity against these strains. We have previously demonstrated that daptomycin at simulated doses of 6 mg/kg/day and 10 mg/kg/day in an *in vitro* pharmacokinetic/pharmacodynamic (PK/PD) model maintained bactericidal activity against some DNS *S. aureus* strains, although time to bactericidal activity was increased and regrowth often occurred (26, 28).

The development of DNS in *S. aureus* has occurred mainly in patients with complicated infections, such as osteomyelitis, septic

arthritis, or endocarditis (6, 32). Optimal therapy for these types of infections requires agents with bactericidal activity to ensure a rapid reduction of the bacterial burden and the highest probability of clinical cure. Unfortunately, clinicians commonly turn to bacteriostatic agents such as linezolid, tigecycline, trimethoprim-sulfamethoxazole, clindamycin, and tetracyclines when they encounter a DNS *S. aureus* infection. Clinical data on successful treatment of infections caused by DNS *S. aureus* is limited to a few case reports (32). The optimal therapy for the treatment of complicated infections caused by DNS *S. aureus* in which a bactericidal regimen is desired is currently unknown. Utilizing the activity daptomycin maintains against DNS strains, we have previously demonstrated a novel combination of daptomycin at 6 mg/kg/day, and trimethoprim-sulfamethoxazole was rapidly bactericidal against two DNS *S. aureus* strains, including one that was also a

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heteroresistant vancomycin-intermediate *Staphylococcus aureus* (hVISA) strain, in a 3-day *in vitro* PK/PD model of simulated endocardial vegetations (SEV) (35). Since our first study utilized only two strains, a relatively short duration of treatment, and the standard dose of daptomycin (6 mg/kg/day), further data are needed to characterize the potential benefit of this novel antibiotic combination. As daptomycin therapy is often salvage therapy after vancomycin and the potential exists under these circumstances to influence daptomycin's activity, it would be prudent to utilize high-dose daptomycin (10 mg/kg) and therefore examine this dose in combination with trimethoprim-sulfamethoxazole over a prolonged period of time in the *in vitro* PK/PD SEV model. Additionally, since the types of infections caused by DNS *S. aureus* strains often require therapy lasting several weeks, the optimal length of combination therapy would need to be evaluated. The objective of the study was to examine the combination of high-dose daptomycin with trimethoprim-sulfamethoxazole for 14 days or de-escalated to daptomycin or trimethoprim-sulfamethoxazole at day 7 against 4 clinical DNS methicillin-resistant *Staphylococcus aureus* (MRSA) strains in a 14-day *in vitro* PK/PD model with SEV.

MATERIALS AND METHODS

Bacterial strains. A total of four clinical DNS MRSA isolates were evaluated: SA H9749-1 (VISA isolate recovered from a patient with osteomyelitis at St. Joseph's Hospital Center, Syracuse, NY), R6212 (hVISA isolate from Detroit Medical Center), R5599 (MRSA blood isolate from Albany Medical Center), and R5563 (MRSA blood isolate).

Antimicrobials. Daptomycin was provided by Cubist Pharmaceuticals. Trimethoprim-sulfamethoxazole was commercially purchased from Sigma-Aldrich Co. (St. Louis, MO). Stock solutions of each antimicrobial were prepared daily per Clinical and Laboratory Standards Institute recommendations (8).

Media. Mueller-Hinton II broth (Difco, Detroit, MI) with 25 mg/liter calcium and 12.5 mg/liter magnesium (MHBII) was used for MIC testing and *in vitro* PK/PD models, except for those with daptomycin. Due to the dependency of daptomycin on calcium for antimicrobial activity, Mueller-Hinton II broth was supplemented (SMHBII) to 50 mg/liter calcium for MIC testing and to 75 mg/liter calcium (equivalent to 50 mg/liter of calcium in the presence of albumin) for *in vitro* PK/PD models (19). Colony counts were determined using tryptic soy agar (TSA; Difco, Detroit, MI) plates. The development of resistance to daptomycin and trimethoprim-sulfamethoxazole was evaluated using either MHBII and agar (Bacto; Difco, Detroit, MI) supplemented with 50 mg/liter of calcium (due to the dependency of daptomycin on calcium for antimicrobial activity) or brain heart infusion agar (BHIA; Difco, Detroit, MI) plates containing the respective antimicrobial at a concentration of three times the MIC.

Susceptibility testing. MICs of study antimicrobial agents were determined by broth microdilution at 10^6 CFU/ml according to Clinical and Laboratory Standards Institute guidelines (8). All were incubated at 37°C for 18 to 24 h.

SEVs. Organism stocks were prepared by inoculating three TSA plates for overnight growth at 37°C. Organisms were swabbed from the growth plates into 5-ml test tubes containing SMHBII, resulting in a concentration of approximately 10^{10} CFU/ml. Simulated endocardial vegetations (SEVs) were prepared in 1.5 ml siliconized Eppendorf tubes by mixing 0.05 ml of organism suspension (final inoculum, 10^9 CFU/g), 0.5 ml of human cryoprecipitated antihemophilic factor from volunteer donors (American Red Cross, Detroit, MI), and 0.025 ml of platelet suspension (platelets mixed with normal saline; 250,000 to 500,000 platelets per clot; American Red Cross, Detroit, MI). Bovine thrombin (0.05 ml; 5,000 U/ml; GenTrac, Inc., Middleton, WI) was added to each tube after inser-

tion of a sterile monofilament line into the mixture. The resultant simulated vegetations were then removed from the Eppendorf tubes with a sterile disposable plastic needle (Becton, Dickinson, Sparks, MD) and introduced into the model. This methodology results in SEVs consisting of approximately 3 to 3.5 g/dl of albumin and 6.8 to 7.4 g/dl of total protein (1).

***In vitro* PK/PD model.** A two-compartment *in vitro* model consisting of a 250-ml glass apparatus with ports where the SEVs were suspended was utilized for all simulations. The apparatus was pre-filled with media, and antibiotics were administered as boluses over a 336-h time period into the central compartment via an injection port. The model apparatus was placed in a 37°C water bath throughout the procedure, and a magnetic stir bar was placed in the media for thorough mixing of the drug in the model. Fresh medium was continuously supplied and removed from the compartment along with the drug via a peristaltic pump (Masterflex; Cole-Parmer Instrument Company, Chicago, IL) set to simulate the half-lives of the antibiotics. Simulated antibiotic regimens were daptomycin at 10 mg/kg every 24 h (estimated total peak and half-life [$T_{1/2}$], 141.1 mg/liter and 8 h, respectively) for 14 days and trimethoprim-sulfamethoxazole at 160/800 mg every 12 h (2.4/100 mg/liter, 10/10 h) for 14 days (4, 20). Combination simulated antibiotic regimens included daptomycin plus trimethoprim-sulfamethoxazole for 14 days, daptomycin plus trimethoprim-sulfamethoxazole for 7 days followed by daptomycin for 7 days, and daptomycin plus trimethoprim-sulfamethoxazole for 7 days followed by trimethoprim-sulfamethoxazole for 7 days. The elimination rate for the combination models was set for the antibiotic with the shorter half-life, and the antibiotic with the longer half-life was supplemented into the second chamber (5). All simulated drug regimens were performed in duplicate to ensure reproducibility.

Pharmacodynamic analysis. Two simulated endocardial vegetations were removed from each model at 0, 4, 8, 24, 32, 48, 72, 96, 120, 144, 168, 176, 192, 200, 216, 240, 264, 288, 312, and 336 h. The SEVs were homogenized and diluted in cold saline to be plated onto TSA plates. To minimize antibiotic carryover, all samples were diluted at least 10-fold before plating. If the drug concentration at the anticipated dilution was within 1 tube dilution of the MIC or higher, then vacuum filtration (100- μ l samples washed through a 0.45- μ m filter with normal saline) was used to avoid antibiotic carryover. Plates were then incubated at 37°C for 24 h, at which time a colony count was performed. These methods have a lower limit of detection of $1 \log_{10}$ CFU/g. The total reduction in \log_{10} CFU/g over 336 h was determined by plotting time-kill curves based on the number of remaining organisms over the 336-h time period. Bactericidal activity (99.9% kill) was defined as a $\geq 3\text{-log}_{10}$ CFU/g reduction in colony count from the initial inoculum. Bacteriostatic activity was defined as a $< 3\text{-log}_{10}$ CFU/g reduction in colony count from the initial inoculum, while inactive was defined as no observed reductions from the initial inoculum. The time to achieve a 99.9% bacterial load reduction was determined by linear regression (if $r^2 \geq 0.95$) or by visual inspection.

Pharmacokinetic analysis. Pharmacokinetic samples were obtained, through the injection port of each model (duplicate samples) at 0, 4, 8, 24, 32, 48, 72, 96, 120, 144, 168, 176, 192, 200, 216, 240, 264, 288, 312, and 336 h for verification of target antibiotic concentrations. All samples were stored at -70°C until they were ready for analysis. Daptomycin concentrations were determined by a previously validated bioassay (*Micrococcus luteus* ATCC 9341; antibiotic assay medium 1; standards of 200, 100, and 50 mg/liter) (2). Blank 1/4-in disks were placed on a preswabbed plate and spotted with 10 μ l of the standard or sample. For trimethoprim, we utilized a bioassay (*S. aureus* R1867; Mueller-Hinton Agar; standards of 40, 20, and 10 mg/liter) (35). Briefly, 1/4-in holes were punched in the preswabbed agar plate and filled with 50 μ l of the standards or samples. This assay has a lower limit of detection of 10 mg/liter and a between-day percent coefficient of variation of 7.15. A separate trimethoprim model was run at 10 times the targeted concentration to utilize this assay due to detection limits. For sulfamethoxazole, we utilized a bioassay (*S. aureus* R1867; Mueller-Hinton agar; standards of 125, 75, and 50 mg/liter) (35).

TABLE 1 MICs for evaluated isolates^a

Isolate	MICs ($\mu\text{g/ml}$)		VAN
	DAP	SXT	
SA H9749-1	4	0.03/0.6	8–16
R6212	4	0.06/1.19	2
R5599	2	0.06/1.19	1
R5563	2	0.03/0.6	2

^a DAP, daptomycin; SXT, trimethoprim-sulfamethoxazole; VAN, vancomycin.

Briefly, 1/4-in holes were punched in the preswabbed agar plate and filled with 50 μl of the standard or sample, which contained 6 mg/liter of trimethoprim. This assay had a between-day coefficient of variation of 9.0%. A separate model was run for sulfamethoxazole to evaluate pharmacokinetic parameters. Each standard was tested in duplicate. Plates were incubated for 18 to 24 h at 37°C, at which time the zone sizes were measured using a protocol reader (Microbiology International, Frederick, MD). The half-lives, areas under the concentration-time curve from 0 to 24 h ($\text{AUC}_{0-24\text{h}}$), $\text{AUC}_{24\text{h}}/\text{MIC}$, 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. Development of resistance was evaluated at 168 and 336 h. One hundred- μl samples from each time point were plated on BHIA or SMHBII agar plates containing three times the drug MIC to assess the development of resistance. Plates were examined for growth after 48 h of incubation at 37°C.

Statistical analysis. Changes in CFU/g at 168 and 336 h were compared by two-way analysis of variance with Tukey's *post hoc* test. 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).

RESULTS

MIC values for the evaluated isolates are displayed in Table 1. All isolates are DNS and trimethoprim-sulfamethoxazole susceptible. Isolate SA H9749-1 is a VISA strain (vancomycin MIC of 8 to 16 $\mu\text{g/ml}$ via broth microdilution), and isolate R6212 was an hVISA strain (ratio with Mu3 of 0.96 by modified population analysis). Observed pharmacokinetic parameters (total peak and $T_{1/2}$) were 145.8 to 146.1 mg/liter (targeted at 141.1 mg/liter) and 6.9 to 8.2 h (targeted at 8 h) for daptomycin, 23.6 to 25.1 mg/liter (targeted at 24 [2.4 mg/liter \times 10]) and 9.3 to 10.1 h (targeted at 10 h) for trimethoprim, and 98 to 104.8 mg/liter (targeted at 100 mg/liter) and 9.6 to 10.2 h (targeted at 10 h) for sulfamethoxazole.

The quantitative changes in the \log_{10} CFU/g for the tested regimens against the four tested strains are displayed in Fig. 1A to D and Table 2. Against all four tested strains, the novel combination of daptomycin plus trimethoprim-sulfamethoxazole for the first 7 days produced a greater than 8 \log_{10} CFU/g reduction by 168 h (7 days), and this decrease was sustained to 336 h (14 days) for the combination regimen and de-escalation to daptomycin at 10 mg/kg or trimethoprim-sulfamethoxazole. The combination of daptomycin plus trimethoprim-sulfamethoxazole produced rapid and sustained bactericidal activity against the VISA strain SA H9749-1 (7 h) and the hVISA strain R6212 (6 h). Time to sustained bactericidal activity with the combination of daptomycin plus trimethoprim-sulfamethoxazole was longer for MRSA R5599 (72 h) and MRSA R5563 (36 h). Trimethoprim-sulfamethoxazole was not bactericidal against any tested strains. Daptomycin alone displayed sustained bactericidal activity against two of the tested strains at 14 h (VISA strain SA H9749-1) and 32 h (MRSA strain

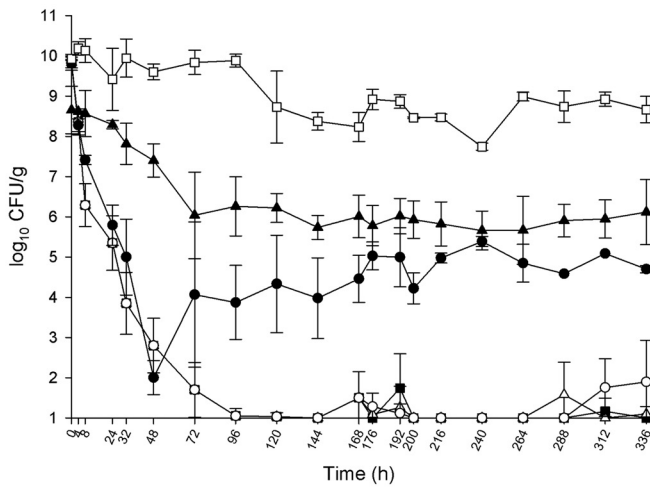
R5563). Daptomycin plus trimethoprim-sulfamethoxazole, the combination de-escalated to daptomycin, and the combination de-escalated to trimethoprim-sulfamethoxazole were significantly better than daptomycin ($P < 0.05$), trimethoprim-sulfamethoxazole ($P < 0.05$), and the growth control ($P < 0.05$) at 168 and 336 h. No organisms with MIC values of 3 \times the initial MIC or greater were recovered for either drug at 168 or 336 h.

DISCUSSION

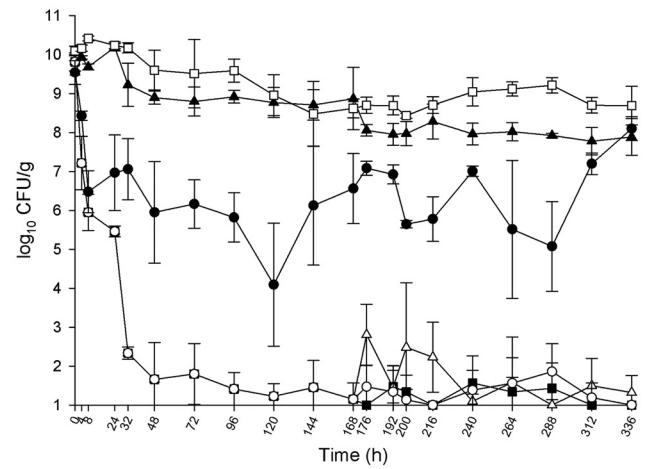
As stated previously, the optimal therapy for DNS MRSA infections is complicated, as the majority are deep-seated, high-inoculum infections requiring long-term therapy, and evidence is limited (6, 21). Optimal therapy would be cost-effective, have minimal side effects, and display bactericidal activity, particularly for endocarditis, in which failure due to bacteriostatic antibiotics is well documented (12, 24). Daptomycin nonsusceptibility in *S. aureus* is a complicated phenotype that can arise from multiple genetic alterations and is variable among strains. The mechanism of DNS in *S. aureus* is due to the additive effects of many potential changes found in some, but not all, strains. One common mechanism is increased cell surface charge, thus repulsing the positively charged daptomycin-calcium complex. This process may occur via translocation of positively charged phospholipids to the outer side of the cytoplasmic membrane, lysinylation of membrane phosphatidylglycerol, and/or increased D-alanylation of cell wall teichoic acids (10, 11, 14, 17, 23, 36). Increased cell wall thickness, alterations in membrane proteins, and changes in membrane fluidity are also often associated with DNS *S. aureus* (10, 16, 22). Since DNS in *S. aureus* is due to the cumulative affect of many of these changes and is not due to one change conferring high-level nonsusceptibility, it is rational that daptomycin retains activity against some of these strains. Previous results from our laboratory have shown that daptomycin maintains variable levels of activity against DNS *S. aureus* (26, 28). The results of the current study were consistent with daptomycin demonstrating delayed bactericidal activity against VISA SA H9749-1 (14 h) and MRSA R5563 (32 h) and a lack of sustained bactericidal activity against the other two strains. The enhancement of this remaining activity in combination with trimethoprim-sulfamethoxazole was examined in this study as a potential combination therapy for DNS MRSA strains.

In this study, we evaluated the activity of daptomycin plus trimethoprim-sulfamethoxazole against four clinical DNS MRSA strains, including one VISA and one hVISA strain, under high-inoculum conditions ($>9.5 \log_{10}$ CFU/g). The combination was able to produce a substantial reduction of greater than 8 \log_{10} CFU/g against all four strains tested. Additionally, for 168 to 336 h (days 7 to 14), the combination, de-escalation to daptomycin, and de-escalation to trimethoprim-sulfamethoxazole were able to sustain this decrease in colony counts and keep viable colony counts close to detection limits. Based on these *in vitro* data, the combination of daptomycin plus trimethoprim-sulfamethoxazole provides a possible treatment option for high-inoculum infections with DNS MRSA, including those in which bactericidal therapy would be optimal. The high susceptibility of *S. aureus* to trimethoprim-sulfamethoxazole of greater than 98% among clinical isolates in the United States supports the potential wide use of this combination (30). Additionally, these *in vitro* data support the de-escalation to either daptomycin or trimethoprim-sulfamethoxazole. This ability to de-escalate while maintaining regimen

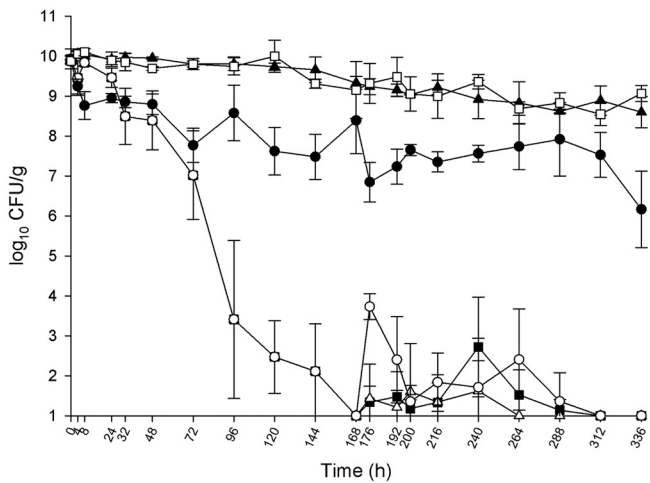
A. SA H9749-1 (VISA)



B. R6212 (hVISA)



C. R5599



D. R5563

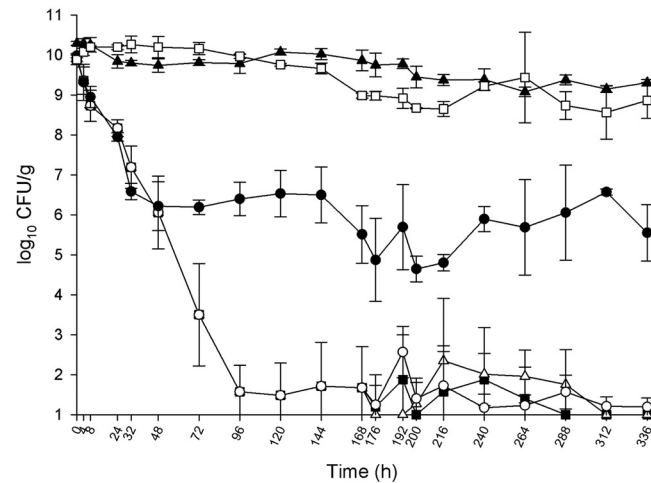


FIG 1 Activity of tested regimens in the *in vitro* pharmacokinetic/pharmacodynamic model of simulated endocardial vegetations. Closed squares, daptomycin plus trimethoprim-sulfamethoxazole (DSXT) for 14 days; open triangle, DSXT for 7 days followed by SXT for 7 days; open circles, DSXT for 7 days followed by daptomycin at 10 mg/kg/day (D10) for 7 days; closed circles, D10 for 14 days; closed triangles, SXT for 14 days; open squares, growth control.

activity provides clinicians with the option to stop one agent if necessary due to cost (daptomycin), side effects (increased serum creatinine with trimethoprim-sulfamethoxazole or increased creatine phosphokinase with daptomycin), or regimen complexity.

Interestingly, time to bactericidal activity was shorter for the VISA (SA H9749-1) and hVISA (R6212) strains than for the two tested MRSA strains R5599 and R5563. While the mechanism for this increased activity is unknown, it is possible the mechanism of

TABLE 2 Activity of tested regimens in the *in vitro* pharmacokinetic/pharmacodynamic model^a

Isolate	Change in log ₁₀ CFU/g at:														
	Time (h) to 99.9% kill					168 h					336 h				
	DAP	SXT	DSXT	-DAP	-SXT	DAP	SXT	DSXT	-DAP	-SXT	DAP	SXT	DSXT	-DAP	-SXT
SA H	14	NA	7	7	7	5.35	2.65	8.34	8.34	8.34	5.11	2.54	8.84	7.94	8.75
R6212	NA	NA	6	6	6	2.98	0.67	8.33	8.33	8.33	1.44	1.65	8.81	8.81	8.49
R5599	336	NA	72	72	72	1.48	0.58	8.93	8.93	8.93	3.7	1.31	8.93	8.93	8.93
R5563	32	NA	36	36	36	4.47	0.42	8.21	8.21	8.21	4.43	0.98	8.88	8.69	8.88

^a Time to 99.9% kill refers to the time to bactericidal activity sustained at 336 h. DAP, daptomycin at 10 mg/kg/day for 14 days; SXT, trimethoprim-sulfamethoxazole for 14 days; DSXT, daptomycin plus trimethoprim-sulfamethoxazole for 14 days; -DAP, daptomycin plus trimethoprim-sulfamethoxazole for 7 days followed by daptomycin for 7 days; -SXT, daptomycin plus trimethoprim-sulfamethoxazole for 7 days followed by trimethoprim-sulfamethoxazole; SA H, SA H9749-1; NA, not applicable.

synergy for daptomycin plus trimethoprim-sulfamethoxazole is more potent against these isolates.

Preliminary clinical data have been favorable regarding this combination to treat osteomyelitis caused by *S. aureus* with reduced susceptibility to both vancomycin and daptomycin (34). The first patient received high-dose daptomycin and trimethoprim-sulfamethoxazole as salvage therapy for vertebral osteomyelitis with SA H9749-1 (vancomycin MIC of 8 to 16 and daptomycin MIC of 4 µg/ml) and achieved a clinical cure. The second patient received daptomycin plus trimethoprim-sulfamethoxazole as salvage therapy for chronic vertebral osteomyelitis with concurrent prolonged bacteremia from SA F31774 (vancomycin MIC of 4 and daptomycin MIC of 4 µg/ml). This combination cleared the bacteremia in 48 h but was stopped after 7 days due to an increase in serum creatinine. The patient's regimen was then changed to daptomycin plus rifampin. Unfortunately, the patient subsequently deteriorated for a week on this combination before dying.

While this study was strengthened by its use of four DNS MRSA strains with various levels of susceptibility to vancomycin and its 14-day study duration, it still has some limitations. While the treatment duration in this *in vitro* model of simulated endocardial vegetations was 14 days, this is still shorter than the 4 to 6 weeks typically required to treat infections such as staphylococcal endocarditis (3). Based on data for the second patient reported above, more than 1 week of combination therapy may be required *in vivo* before de-escalation. In conclusion, the combination of daptomycin plus trimethoprim-sulfamethoxazole produced sustained bactericidal activity against four DNS MRSA strains, including one VISA strain and one hVISA strain, and this inoculum decrease was maintained when de-escalating to daptomycin or trimethoprim-sulfamethoxazole. Further clinical research examining this combination and the optimal time to de-escalate is warranted.

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