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Journal

Antimicrobial Agents and Chemotherapy, 64(3)

ISSN

0066-4804

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Publication Date

2020-02-21

DOI

10.1128/aac.02072-19

Peer reviewed

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EXPERIMENTAL THERAPEUTICS

Ability of Bicarbonate Supplementation To Sensitize Selected Methicillin-Resistant *Staphylococcus aureus* Strains to β-Lactam Antibiotics in an *Ex Vivo* Simulated Endocardial Vegetation Model

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ABSTRACT Supplementation of standard growth media (cation-adjusted Mueller-Hinton Broth [CAMHB]) with bicarbonate ($NaHCO_3$) increases β-lactam susceptibility of selected methicillin-resistant *Staphylococcus aureus* (MRSA) strains ("$NaHCO_3$ responsive"). This "sensitization" phenomenon translated to enhanced β-lactam efficacy in a rabbit model of endocarditis. The present study evaluated $NaHCO_3$-mediated β-lactam MRSA sensitization using an *ex vivo* pharmacodynamic model, featuring simulated endocardial vegetations (SEVs), to more closely mimic the host microenvironment. Four previously described MRSA strains were used: two each exhibiting *in vitro* $NaHCO_3$-responsive or $NaHCO_3$-nonresponsive phenotypes. Cefazolin (CFZ) and oxacillin (OXA) were evaluated in CAMHB with or without $NaHCO_3$. Intra-SEV MRSA killing was determined over 72-h exposures. In both "responsive" strains, supplementation with 25 mM or 44 mM $NaHCO_3$ significantly reduced β-lactam MICs to below the OXA susceptibility breakpoint (≤4 mg/liter) and resulted in bactericidal activity (≥3-log killing) in the model for both OXA and CFZ. In contrast, neither *in vitro*-defined nonresponsive MRSA strain showed significant sensitization in the SEV model to either β-lactam. At both $NaHCO_3$ concentrations, the fractional time above MIC was >50% for both CFZ and OXA in the responsive MRSA strains. Also, in media containing RPMI plus 10% Luria-Bertani broth (proposed as a more host-mimicking microenvironment and containing 25 mM $NaHCO_3$), both CFZ and OXA exhibited enhanced bactericidal activity against $NaHCO_3$-responsive strains in the SEV model. Neither CFZ nor OXA exposures selected for emergence of high-level β-lactam-resistant mutants within SEVs. Thus, in this *ex vivo* model of endocarditis, in the presence of $NaHCO_3$ supplementation, both CFZ and OXA are highly active against MRSA strains that demonstrate similar enhanced susceptibility in $NaHCO_3$-supplemented media *in vitro*.

KEYWORDS MRSA, bicarbonate, *Staphylococcus aureus*, beta-lactams, bicarbonate, methicillin resistance

Methicillin-resistant *Staphylococcus aureus* (MRSA) is an important human pathogen that is associated with both community-onset and nosocomial-onset infections. Despite development of newer anti-MRSA antibiotics over the past decades (e.g., daptomycin, linezolid, oritavancin, dalbavancin, and ceftaroline) (1, 2), therapeutic issues with these agents, such as emergence of resistance or toxicities, have

Citation Rose WE, Bienvenida AM, Xiong YQ, Chambers HF, Bayer AS, Ersoy SC. 2020. Ability of bicarbonate supplementation to sensitize selected methicillin-resistant *Staphylococcus aureus* strains to β-lactam antibiotics in an *ex vivo* simulated endocardial vegetation model. Antimicrob Agents Chemother 64:e02072-19. https://doi.org/10.1128/AAC.02072-19.

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Received 15 October 2019
Returned for modification 6 November 2019
Accepted 8 December 2019
Accepted manuscript posted online 16 December 2019
Published 21 February 2020

TABLE 1 Cefazolin and oxacillin MICs in standard and alternative medium types used in the pharmacodynamic model

Medium or media	MIC (mg/liter)							
	CFZ				OXA			
	11-11	MW2	COL	BMC-1001	11-11	MW2	COL	BMC-1001
CAMHB-Tris	16	8	256	256	64	32	512	256
CAMHB-Tris + 2% NaCl	16	8	256	256	64	32	512	256
CAMHB-Tris + 25 mM $NAHCO_3$	4	8	256	256	4	32	512	256
CAMHB-Tris + 44 mM $NAHCO_3$	1	1	256	256	1	2	512	256
RPMI 1640 + 10% LB medium	1	2	32	8	1	1	64	4

often limited their utility (3–5). In turn, clinicians continue to rely on older anti-MRSA agents with proven efficacy (vancomycin), often employing multidrug combination treatments (6).

One of the limitations of antibiotic use is the predictive value of antimicrobial susceptibility testing and breakpoint determinations for selection. Although other diagnostics for infectious diseases have rapidly evolved over the past decades, antimicrobial susceptibility testing for MRSA has largely remained unchanged since the 1960s, with the use of bacterial growth medium such as Mueller-Hinton broth and agar (MHB and MHA). For *S. aureus* susceptibility testing with oxacillin (OXA), the Clinical and Laboratory Standards Institute (CLSI) recommends growth of bacteria in 2% NaCl cation-adjusted MHB (CAMHB) (7, 8). Although the latter medium will amplify the capacity to isolate OXA-resistant subpopulations within MRSA strains, it does not accurately reflect the host milieu; thus, the resulting MICs may not accurately represent antibiotic activity against MRSA within host-specific microenvironments (9).

Recent studies have focused on refining *in vitro* growth media to better simulate the host microenvironment *in vivo* in the context of more relevant and translatable antimicrobial susceptibility testing. Supplementation of standard media with bicarbonate ($NaHCO_3$), a ubiquitous buffer in humans (normally in the range of 23 to 29 mmol/liter *in vivo*), has become the subject of several such studies (9, 10). These reports have demonstrated the ability of $NaHCO_3$ supplementation to augment the susceptibility of selected MRSA strains *in vitro* to β-lactams. These investigations have focused on two conventional, prototype β-lactams not recommended for use against MRSA: OXA and cefazolin (CFZ) (10). These data enabled identification of two distinct MRSA phenotypes, "$NaHCO_3$ responsive" and "$NaHCO_3$ nonresponsive." These *in vitro* phenotypes accurately predicted the ability of these same β-lactams to clear MRSA from multiple target tissues in a rabbit model of MRSA infective endocarditis (i.e., cardiac vegetations, kidneys, and spleen) (10). The mechanism(s) by which $NaHCO_3$ sensitizes "responsive" strains to such β-lactams appear to involve, at least in part, multiple genes which are critical in maintaining the MRSA phenotype, such as *mecA* and *sarA* (8); these perturbations may, in turn, lead to decreased production and/or maturation of PBP2a, yielding a "functional MSSA" phenotype (10).

The present study expands on our previous findings of bicarbonate sensitization of MRSA to β-lactams using a pharmacodynamic model featuring *ex vivo* simulated endocardial vegetations (SEVs). We hypothesized that "bicarbonate responsiveness" in MRSA in this model would mirror similar findings in the rabbit endocarditis model and that host-mimicking media within SEVs would help identify novel pharmacodynamic optimization strategies for prototypical β-lactams against such $NaHCO_3$-responsive MRSA strains.

RESULTS

β-Lactam susceptibilities in standard and alternative media supplemented with $NaHCO_3$. CFZ and OXA MICs have been previously reported for these four study strains (10). In this investigation, we confirmed the strain-dependent $NaHCO_3$ enhancement of the susceptibility of these isolates to the two study β-lactams. As noted in Table 1, strains 11-11 (USA300; ST8 and SCC*mec* IV) and MW2 (USA400; ST1 and SCC*mec*

TABLE 2 Pharmacokinetics of CFZ and OXA in the central compartment (medium) of the pharmacodynamic model[a]

Parameter	CFZ, 2 g every 8 h		OXA, 2 g every 6 h	
	Predicted	Observed ($n = 12$)	Predicted	Observed ($n = 12$)
C_{max} (mg/liter)	256	249.4 ± 2.7	150	152.1 ± 1.4
C_{min} (mg/liter)	16	15 ± 1	2.3	2.2 ± 0.1
k_e (h^{-1})	0.385	0.354 ± 0.032	0.693	0.705 ± 0.005
Half-life (h)	1.8	2.0 ± 0.2	1	1.0 ± 0.0[b]
AUC_{0-24} (mg/liter · h)	2,442	2,384 ± 31	1162	1,217 ± 91

[a]Data for observed values are presented as means ± the standard errors.
[b]The standard error for this value was <0.01.

IV) displayed a significant reduction in β-lactam MICs in media supplemented with $NaHCO_3$ (44 mM), while β-lactam susceptibility was not affected with $NaHCO_3$ supplementation for the other two strains, COL (USA100; ST250 and SCC*mec* I) and BMC-1001 (USA500; ST8 and SCC*mec* IV). In the two $NaHCO_3$-responsive strains, supplementation with 44 mM $NaHCO_3$ reduced the CFZ MIC to below the OXA susceptibility breakpoint (≤4 mg/liter), with an 8- to 16-fold MIC reduction. Similarly, for these same strains, the OXA MICs were reduced 16- to 64-fold with 44 mM $NaHCO_3$ supplementation.

It has recently been reported that an "antibiotic sensitization" effect, especially for Gram-negative bacteria and selected β-lactams, can occur in other host-mimicking media (9–11). The standard cell culture medium, RPMI 1640, contains physiologic concentrations of $NaHCO_3$ (~25 mM). In this medium, as opposed to CAMHB, we noted substantially increased β-lactam susceptibility in all four strains regardless of genotypic background. However, the two $NaHCO_3$-responsive strains were generally more responsive in this host-mimicking media, with resultant β-lactam MICs of ≤2 mg/liter (Table 1), which is consistent with previous findings (10).

β-Lactam pharmacokinetics in the *ex vivo* SEV model. The pharmacokinetic profiles of antibiotics within the *ex vivo* SEV model is computer designed to precisely mimic actual patient exposures clinically. Table 2 provides the predicted versus observed pharmacokinetic profiles of CFZ and OXA in the SEV model's central "fluid" compartment (media; see Fig. 4). We simulated high-dose CFZ administration (2 g every 8 h) and OXA (2 g every 6 h), as recommended for invasive MSSA infections (12, 13). Also, these dose regimens approximate those used in our prior rabbit endocarditis study with these same strains (14). The concentrations achieved in the *ex vivo* model closely correlated to the targeted parameters for both antibiotics.

Antimicrobial activity in the *ex vivo* SEV model. For the antibiotic activities in the *ex vivo* SEV pharmacodynamic model, see Fig. 1 and 3. Table 3 compares the ability of each β-lactam to reduce the MRSA counts within the SEVs over the 72-h course of treatment (expressed as the area under the bacterial curves [AUBC]). As a point of reference,

TABLE 3 Area under the bacterial curve of CFZ and OXA treatment in standard and alternative media[a]

Regimen	Medium or media	Mean AUBC ± SD			
		11-11	MW2	COL	BMC-1001
CFZ	CAMHB-Tris (control)	626.2 ± 6.6	658 ± 3.0	648.4 ± 2.8	633.9 ± 4.5
	CAMHB-Tris + 25 mM $NAHCO_3$	455.3 ± 4.8[b]	489.2 ± 2.5[b]	612.1 ± 2.5	592.7 ± 3.4
	CAMHB-Tris + 44 mM $NAHCO_3$	310.0 ± 10.5[b,c]	390.8 ± 4.8[b,c]	601.9 ± 4.5	566.7 ± 2.9[b]
	RPMI 1640 + 10% LB medium	378.3 ± 3.5[b,c]	387.3 ± 6.9[b,c]	505.3 ± 8.1[b]	538.8 ± 6.3[b]
OXA	CAMHB-Tris	610 ± 3.5	661.9 ± 9.5	621.1 ± 3.6	642.4 ± 5.1
	CAMHB-Tris + 25 mM $NAHCO_3$	480 ± 6.3[b]	539.2 ± 23.3[b]	596.7 ± 6.7	617.3 ± 2.6
	CAMHB-Tris + 44 mM $NAHCO_3$	392 ± 17.8[b,c]	399.4 ± 15.0[b,c]	568.9 ± 11.0[b]	603.0 ± 538
	RPMI 1640 + 10% LB medium	415.1 ± 4.2[b,c]	438.1 ± 9.3[b,c]	649.7 ± 4.7	599.1 ± 9.4

[a]The area under the bacterial curve (AUBC) is inversely related to antibiotic activity, with lower AUBC indicating greater antibiotic effect. The data represent means ± the standard deviations of duplicate replicates, with two samples taken at each time point ($n = 4$). CAMHB-Tris includes 2% NaCl.
[b]$P < 0.05$ versus control.
[c]$P < 0.05$ versus 25 mM $NAHCO_3$.

the less the AUBC, the more active the antibiotic regimen (14). The following trends emerged from these studies:

(i) The β-lactams were inactive against all MRSA in the *ex vivo* SEV model with CAMHB in the central compartment without bicarbonate supplementation. As expected, based on the intrinsic MICs, neither CFZ nor OXA had a substantive microbiologic effect against the four strains in the SEV model at human-equivalent, pharmacokinetic-based simulated dose-regimens. The activity curves among the strains were indistinguishable regardless of clonal background at all time points (Fig. 1 and 2), as well as for the overall exposure based on similar AUBC values (Table 3). Based on the MICs determined in CAMHB, OXA achieved zero percent *f*T>MIC (time above MIC of the free drug) for all strains, while CFZ achieved zero percent *f*T>MIC for COL and BMC-1001 and 33 and 57% *f*T>MIC for 11-11 and MW2, respectively.

(ii) Bicarbonate supplementation of CAMHB in the central compartment resulted in significant CFZ or OXA bactericidal activity in responsive (but not in nonresponsive) MRSA strains in the *ex vivo* SEV model. Figure 1 displays the pharmacodynamic SEV kill curve of CFZ against all four strains, with or without $NaHCO_3$ supplementation. Based on MIC reductions noted in strains 11-11 and MW2 in this study, as well as in our previous *in vitro* work (14), we predicted that CFZ and OXA would each yield substantial intra-SEV antimicrobial activity against these responsive strains in the presence of $NaHCO_3$ supplementation. Accordingly, in the *ex vivo* SEV model supplemented with $NaHCO_3$, CFZ and OXA resulted in significantly greater anti-MRSA activity compared to standard CAMHB medium in these responsive strains. After 8 h in the SEV model, β-lactam exposures in bicarbonate-supplemented media resulted in significantly greater activity compared to standard CAMHB media (Fig. 1, $P < 0.05$ for time points 8 to 72 h). In comparing *ex vivo* kill curves between the different $NaHCO_3$ concentrations, β-lactam exposure of the "responsive strains" in CAMHB medium supplemented with 44 mM $NaHCO_3$ yielded a significantly greater bacterial count reduction for both CFZ and OXA versus 25 mM $NaHCO_3$ supplementation at most time points from 24 to 72 h (Fig. 1 and 2, $P < 0.05$). In contrast, there was no difference in killing of the nonresponsive strains, COL and BMC-1001, with $NaHCO_3$ supplementation (versus either antibiotic-containing standard CAMHB medium or in untreated growth controls) ($P > 0.05$).

Overall, there was a notable $NaHCO_3$ concentration response, with higher bacterial count reductions, faster time to bactericidal activity (i.e., the time to a $\geq$3 $\log_{10}$ CFU/g reduction), and lower AUBC when media were supplemented with 44 mM versus 25 mM $NaHCO_3$ (Fig. 1 and Table 3). This correlated with greater susceptibility with the higher $NaHCO_3$ concentration, resulting in 100% *f*T>MIC for both strains 11-11 and MW2 with 44 mM $NaHCO_3$ versus 57 and 83% *f*T>MIC, respectively, with 25 mM $NaHCO_3$ for these strains. Since no change in MIC occurred in CAMHB plus $NaHCO_3$ in the nonresponsive strains, the *f*T>MIC remained at zero percent, and this reflects the lack of any β-lactam activity against those strains.

CFZ and OXA are each bactericidal *ex vivo* against $NaHCO_3$ responsive (but not against nonresponsive) MRSA in the host-mimicking medium, RPMI. As noted above, the CFZ and OXA MICs for all four strains were substantially reduced in RPMI. We next determined whether these *in vitro* outcomes were mirrored *ex vivo* in the SEV model. As displayed in Fig. 3, the $NaHCO_3$-responsive strains 11-11 and MW2 were significantly killed with CFZ or OXA treatment in this medium; this is reflected by the enhanced pharmacodynamic attainment in this medium, resulting in 100% *f*T>MIC for CFZ and 60% *f*T>MIC for OXA against both 11-11 and MW2 strains. In contrast, over the same 72-h β-lactam exposure period, the two nonresponsive strains (COL and BMC-1001) were minimally affected by either agent. It should be noted that there was initial activity with CFZ against COL in RPMI, with $\sim$2 $\log_{10}$ CFU/g killing at 24 to 48 h. However, this was not sustained after 48 h, and the strain regrew to the initial inoculum. These data are reflected in the lower target attainment of 8.2 to 56% *f*T>MIC for CFZ and 0 to 20% for *f*T>MIC for OXA.

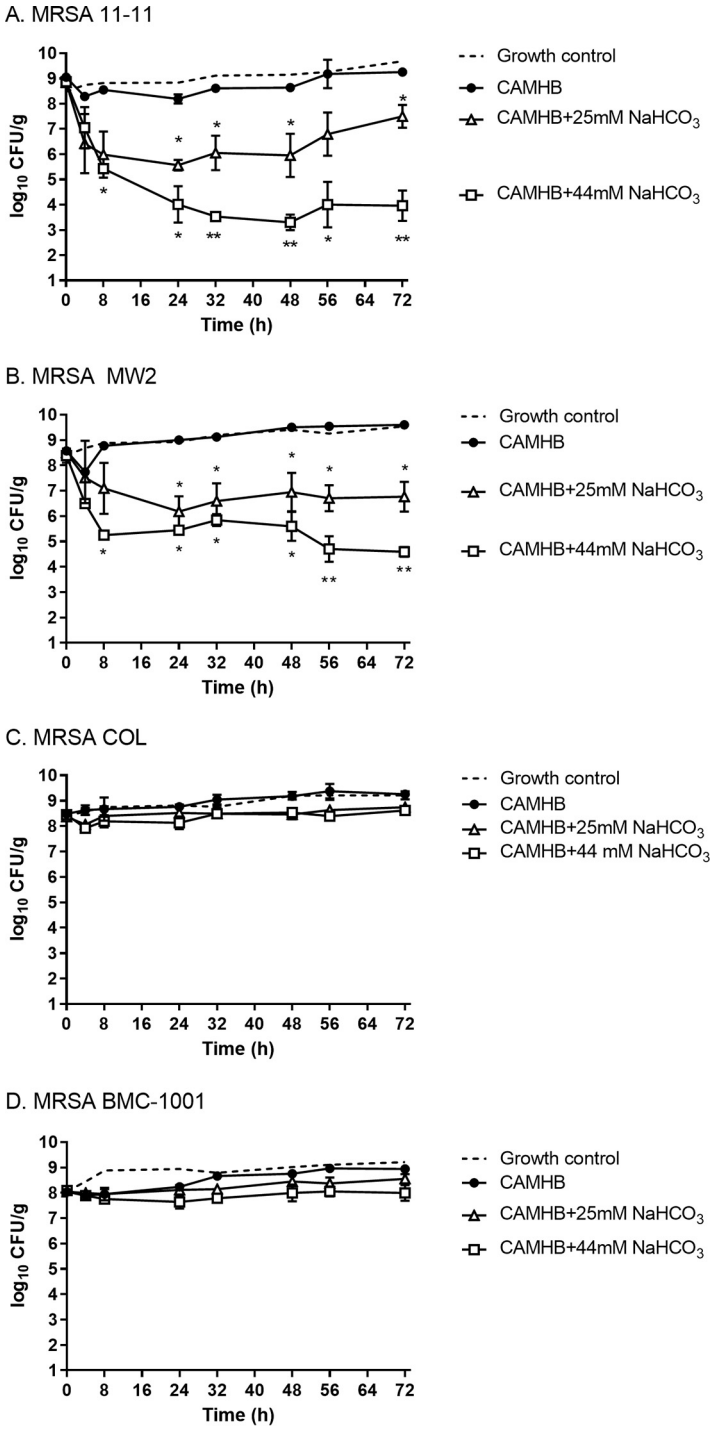

FIG 1 Kill curve activity of simulations for CFZ administered at 2 g every 8 h in the *ex vivo* SEV model in standard media and in $NaHCO_3$-supplemented media against MRSA strains 11-11 (A), MW2 (B), COL (C), and BMC-1001 (D). The dashed line indicates untreated growth in CAMHB-Tris, and the solid lines indicate CFZ regimens. *, $P < 0.05$ versus CAMHB; **, $P < 0.05$ versus CAMHB plus 25 mM $NaHCO_3$ media.

β-Lactam treatment did not select for high-level CFZ or OXA resistance regardless of the $NaHCO_3$ responsivity phenotype. One additional advantage of this *ex vivo* model system is the ability to screen for emergence of drug-resistant mutants during human-simulated treatment strategies. In all our SEV simulations, we screened for evolution of high-level β-lactam-resistant mutants (≥4× the initial MIC) at time zero versus every

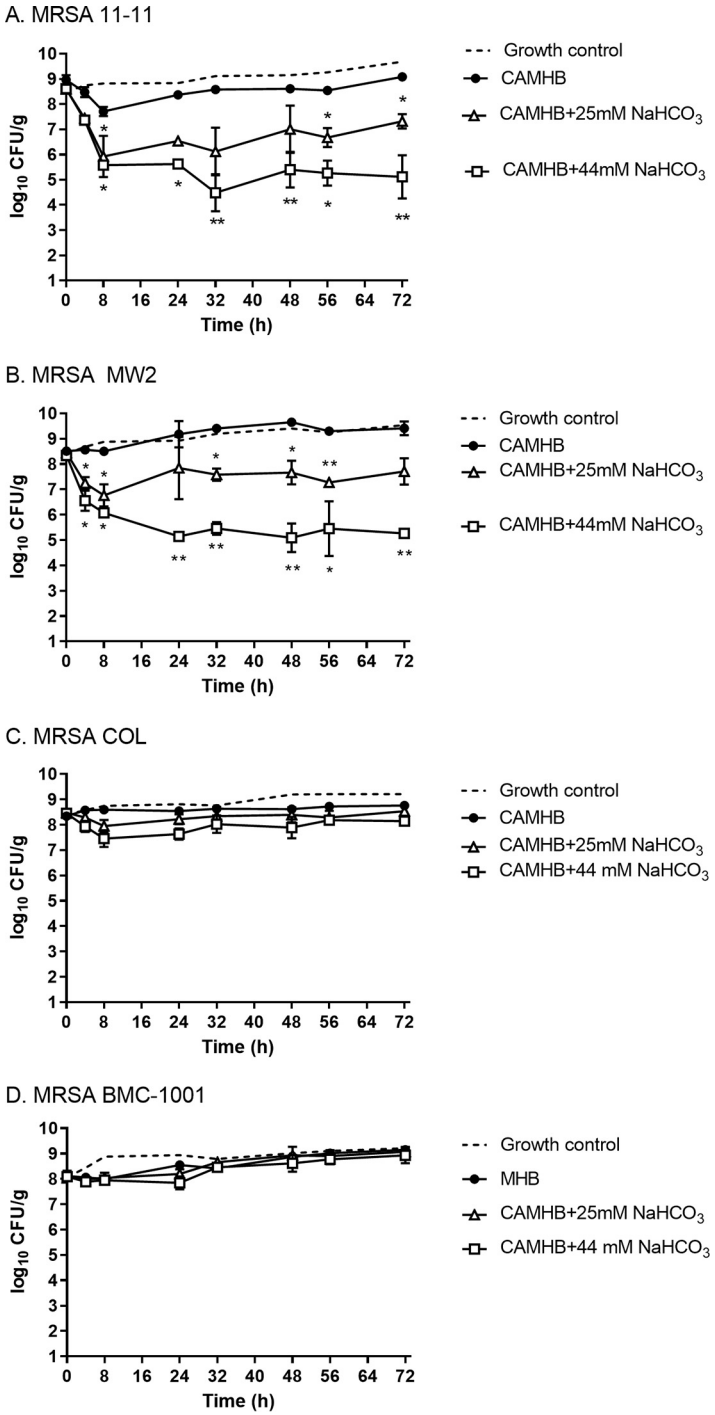

FIG 2 Kill curve activity of simulations for OXA administered at 2 g every 6 h in the *ex vivo* SEV model in standard media and $NaHCO_3$-supplemented media against MRSA strains 11-11 (A), MW2 (B), COL (C), and BMC-1001 (D). The dashed line represents untreated growth in CAMHB-Tris; the solid lines indicate OXA regimens. *, $P < 0.05$ versus CAMHB; **, $P < 0.05$ versus CAMHB plus 25 mM $NaHCO_3$ media.

24 h thereafter. No such resistant variants were confirmed over the course of treatment with either CFZ or OXA.

DISCUSSION

The β-lactam class of antibiotics remains the treatment of choice for a broad range of susceptible pathogens due to their potent and rapid mechanism(s) of action, as well

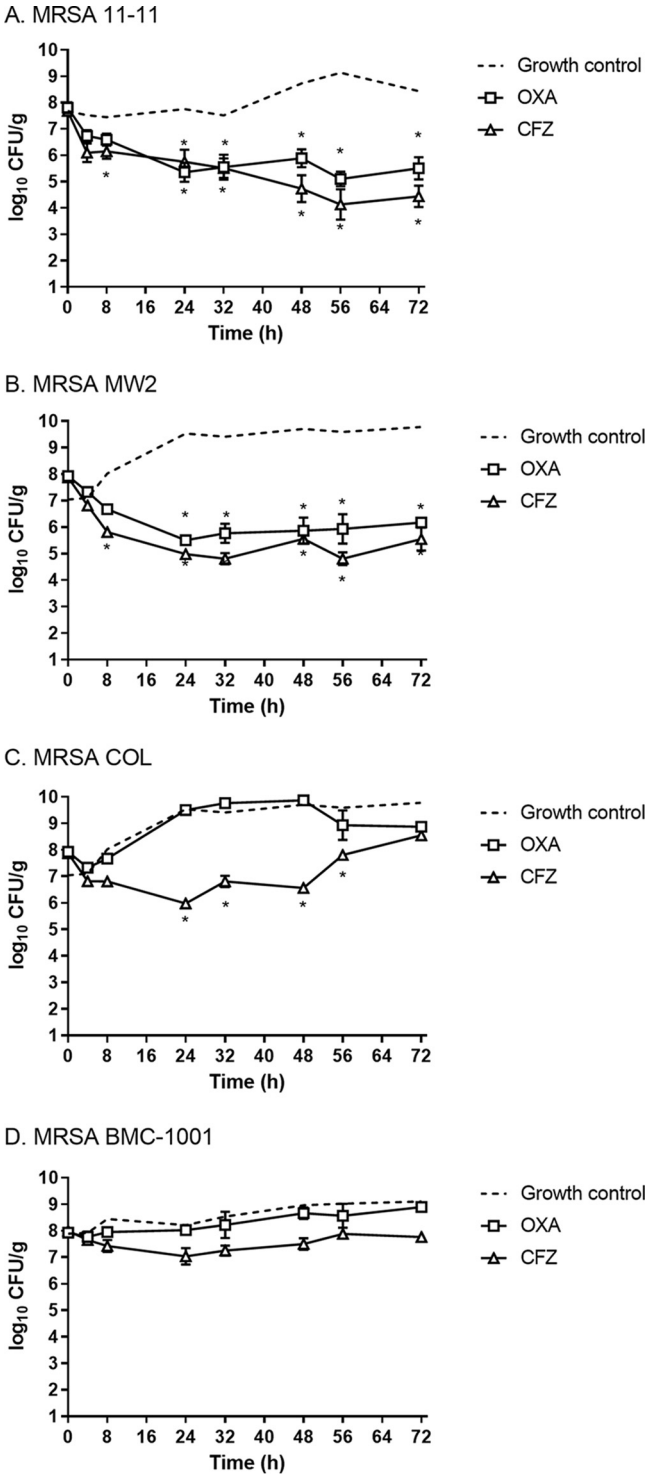

FIG 3 Kill curve activity of simulations for CFZ administered at 2 g every 8 h and OXA administered at 2 g every 6 h in an *ex vivo* SEV model in host-mimicking RPMI–10% LB media against MRSA strains 11-11 (A), MW2 (B), COL (C), and BMC-1001 (D). *, $P < 0.05$ versus control.

as their relatively low rates of adverse side effects and toxicities. In addition, they have recently become well characterized as able to augment the innate host immune system via their synergistic interactions with host defense peptides against key Gram-positive pathogens, including *S. aureus* (15–17).

The present study extends our previous work on the ability of $NaHCO_3$ supplemen-

tation of standard MRSA *in vitro* media to identify a subset of MRSA strains that may, in fact, exhibit β-lactam susceptibility (10, 18, 19). The notion of using alternative media for antibiotic susceptibility screening is not new; however, the biological basis and clinical utility of this approach has substantially increased in the last few years in a variety of bacteria, including *Salmonella*, *Acinetobacter*, and *Staphylococcus* spp. (9, 11, 19). It should be emphasized that all four of our prototype MRSA isolates were rendered significantly more β-lactam susceptible *in vitro* in RPMI media (4- to 64-fold MIC reductions). However, these salutary *in vitro* results in RPMI did not accurately predict subsequent microbiologic outcomes in the SEV model.

The enhanced antimicrobial activity seen above in such alternative media, as well as their outcome predictability *in vivo* may, in part, reflect (i) the enhanced activity of host defense peptides in combination with antibiotics *in vivo*, (ii) bacterial adaptations *in vivo* that prevent excessive microbial growth, and/or (iii) blunted expression of antimicrobial resistance mechanisms (10, 11, 16). However, it should be noted that some host-mimicking environments may actually be beneficial for bacterial survival. For example, under more acidic conditions, *Escherichia coli* may become more resistant to β-lactams due to the higher PBP1b production needed for organism survival in this harsh environment (20).

The treatment of MRSA infections poses several daunting challenges. Current treatment options for MRSA infections are relatively limited and include antibiotics that are not only costly but also substantially less effective and often more toxic compared to standard antibiotic treatments for MSSA (21–23). This is in large part due to the perceived inability to use traditional β-lactam antibiotics for MRSA infections (2). The *mecA* gene is primarily responsible for mediating *S. aureus* resistance to traditional β-lactams, encoding penicillin-binding protein 2A (PBP2A), which has low affinity to most standard-of-care antistaphylococcal β-lactam antibiotics (2). Despite this, combination therapy featuring β-lactams plus anti-MRSA agents such as vancomycin and daptomycin has proven to be effective in selected patients with recalcitrant MRSA infections (15, 24–27). In addition, many MRSA strains that exhibit reduced susceptibility to vancomycin and daptomycin often demonstrate a paradoxical increase in susceptibility to β-lactams, a phenomenon known as the "see-saw effect"; this provides an additional scenario in which β-lactam agents may provide synergistic efficacy for MRSA killing (15).

Following our discovery of the $NaHCO_3$-responsive phenotype in several prototype MRSA strains in terms of β-lactam hypersusceptibility *in vitro* (10), we recently screened a large collection of well-characterized MRSA strains ($n = 58$) for this same phenotype. We identified that approximately three-quarters of this cohort were CFZ susceptible in $NaHCO_3$-supplemented CAMHB, whereas approximately one-third were OXA susceptible in the same media (28). The more active effect of CFZ in our *ex vivo* SEV model, as well as in the rabbit endocarditis model (10), would support this finding.

We investigated the potential mechanisms by which $NaHCO_3$ may cause such β-lactam hypersusceptibility *in vitro* and demonstrated that $NaHCO_3$-supplemented media can (i) downregulate expression of the *mecA* gene and subsequent PBP2a production and (ii) blunt the expression of several genes involved in maintenance of the MRSA phenotype, including *sarA* and *blaZ* (10). In MRSA, PBP4 is also essential for peptidoglycan cross-linking (29), so it will be of interest to determine whether $NaHCO_3$ responsiveness in selected MRSA strains might result from the downregulation of PBP4 expression and reduction in that protein's production. This additional potential mechanism is under investigation.

The pharmacodynamic parameter best correlated with efficacy of β-lactam antibiotics is *f*T>MIC; this metric has been highly studied *in vivo* and *in vitro* and has been validated to predict improved clinical outcomes in β-lactam-treated patients (30–33). In most investigations, β-lactams demonstrate optimal activity with a "target attainment" of *f*T>MIC in the range of 40 to 60% (30). Relevant to our study, we found that the lower MICs observed in $NaHCO_3$-supplemented media for two of our prototype strains—11-11 and MW2—resulted in an "MSSA-like phenotype," improving the po-

tential for target attainment for both CFZ and OXA. For the $NaHCO_3$-responsive strains, CFZ achieved at least 83% *f*T>MIC, resulting in a substantial and durable bactericidal activity in the *ex vivo* SEV model. Similarly, OXA, with a calculated ~60% *f*T>MIC in the two $NaHCO_3$-responsive strains, also achieved good bactericidal activity in the *ex vivo* model. The effect of higher doses of oxacillin as recommended for humans with endocarditis (≥12 g/day), as well as alternative infusion strategies (such as continuous infusion) are being explored to optimize this $NaHCO_3$-responsive phenotype in our SEV model.

The *ex vivo* SEV model has been extensively used to pharmacokinetically and pharmacodynamically study the impacts of many antimicrobials against a number of clinical bacterial isolates; these investigations have focused on verifying these organism-antimicrobial interactions in a setting more akin to the host tissue microenvironment than standard *in vitro* assays (34, 35). In addition, this model has been shown to successfully recapitulate the microbiologic results generated in several *in vivo* animal models, including rabbit endocarditis (36). In the present investigation, we used this same *ex vivo* pharmacokinetic/pharmacodynamic model to more systematically evaluate the activity of CFZ and OXA against our four prototype MRSA isolates in a host-mimicking microenvironment. Our current results in the SEV model further strengthen an important "bridge" between *in vitro* and *in vivo* outcomes with these strains. First, the two MRSA strains that were significantly β-lactam/$NaHCO_3$ responsive *in vitro* and *in vivo* (10) showed enhanced intra-SEV killing by CFZ and OXA in the presence of $NaHCO_3$. In parallel, the two β-lactam/$NaHCO_3$-nonresponsive strains (as defined *in vitro* and *in vivo*) were not substantially killed by CFZ or OXA *ex vivo*. Second, as seen *in vitro*, there appeared to be a $NaHCO_3$ concentration optimum for the β-lactam/$NaHCO_3$-sensitizing phenotype for the two responsive strains (11-11 and MW2). Thus, significant killing was seen *ex vivo* within SEVs both at 25 and 44 mM, but with a substantially greater bactericidal effect seen at the latter concentration. In contrast, the excellent *in vivo* clearance of both $NaHCO_3$-responsive MRSA which occurs *in vivo* at $NaHCO_3$ concentrations of 20 to 25 mM (10) suggests that additional host factors within cardiac vegetations or other target organs are in play to synergistically kill MRSA (e.g., higher tissue levels of $NaHCO_3$ than blood levels or additive antimicrobial actions of polymorphonuclear leukocytes, platelets, host defense peptides, and/or; antibody, etc. [9–11]).

In conclusion, this study further validates the potential clinical translatability of the intriguing finding of β-lactam/$NaHCO_3$ sensitization of selected MRSA strains *in vitro*. It will be important to extend our studies to even larger clinical MRSA cohorts (28). Ultimately, a pivotal human trial will be required to fully adjudicate the clinical utility of defining MRSA strains as β-lactam/$NaHCO_3$ responsive in the clinical microbiology laboratory. There may be supportive data to justify this in the literature, e.g., the equivalent efficacy of the β-lactam, cephalexin, versus clindamycin in a randomized controlled clinical trial for MRSA skin infections (37) (despite cephalexin resistance by traditional *in vitro* susceptibility testing). These prior studies provide support for an ultimate clinical trial to assess the notion of β-lactam/$NaHCO_3$ responsiveness in MRSA.

MATERIALS AND METHODS

Bacterial strains, media, and antibiotics. The strains used in this study were all clinical MRSA isolates and represent diverse contemporary clonal genotypes (USA types) found worldwide in MRSA infections. These included MRSA 11-11 (USA300), MW2 (USA400), COL (USA100), and BMC-1001 (USA500). These strains are well described in the literature and were recently utilized to define $NaHCO_3$ responsivity *in vitro* (10). All strains were stored at −80°C in tryptic soy broth with 15% glycerol until thawed for use. Bacteria were cultured on MHA and incubated at 37°C in ambient air. Liquid culture medium used for bacterial growth in susceptibility testing, and pharmacodynamic modeling included four different types: (i) cation-adjusted Mueller-Hinton broth (CAMHB; Difco) with the addition of 100 mM Tris (hydroxymethyl-aminomethane; Fisher Scientific) to maintain pH at approximately 7.3 ± 0.1, (ii) CAMHB-Tris supplemented with 25 mM $NaHCO_3$, (iii) CAMHB-Tris supplemented with 44 mM $NaHCO_3$, or (iv) tissue culture medium, Roswell Park Memorial Institute (RPMI) 1640 (Fisher Scientific) supplemented with 10% Luria-Bertani (LB) broth. The latter medium contains ~25 mM $NaHCO_3$, as well as biotin, vitamin B_{12}, and PABA, as well as vitamins, inositol, and choline. Also, all four liquid medium types were supplemented with 2% NaCl as recommended by the CLSI. The β-lactam antibiotics OXA and CFZ were

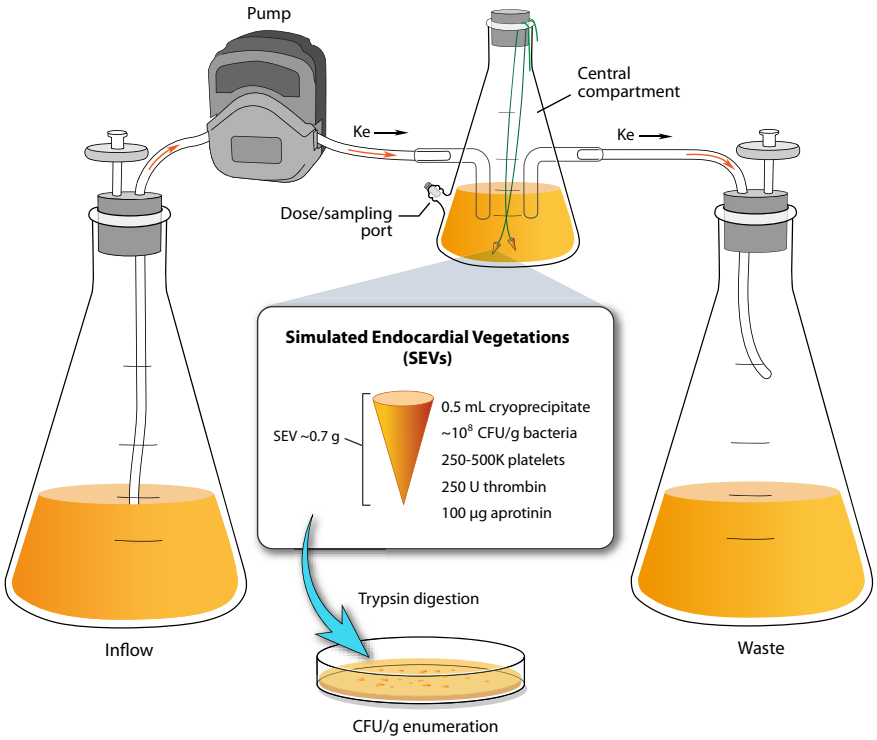

FIG 4 Illustration of the *ex vivo* simulated endocardial vegetation model components and design.

purchased as analytical powders from Sigma-Aldrich (St. Louis, MO) and prepared fresh prior to each experiment according to the manufacturer's protocols.

Antibiotic susceptibility assays. CLSI guidelines (7) for broth microdilution were used to determine antibiotic susceptibilities (MICs) with modifications to the recommended media (8). Bacteria were grown overnight at 35°C on MHA, and resulting colonies were suspended in the different media listed above to the equivalent of 0.5 McFarland standard. Samples were further diluted 1:100 for a final inoculum of 5×10^5 CFU/ml. Antibiotics were serially diluted 2-fold, and MICs were defined by standard metrics (7) and determined in triplicate on two separate days ($n = 6$ replicates).

Pharmacodynamic model with *ex vivo* SEVs. The SEV model components and characteristics are illustrated in Fig. 4. Pooled human cryoprecipitate antihemophilic factor prepared from plasma (fibrinogen, von Willebrand factor, factor VIII, factor XIII, and fibronectin), and pooled platelets were collected from human volunteer donors (UW Health Blood Bank, Madison, WI). C Bovine thrombin (UW Health) and aprotinin (Sigma-Aldrich) were commercially purchased. This preparation results in SEVs containing 3 to 3.5 g/dl of albumin and 6.8 to 7.4 g/dl of total protein (equating to human physiologic levels) (17, 34). The protein binding of the study drugs has been found to be 84% for CFZ and 92% for OXA, which was used to calculate the free AUC (fAUC) and the percent time of free drug above the MIC (%fT>MIC) (38, 39).

The central ("fluid") compartment model for the SEVs consists of a 150-ml flask, which was prefilled with media and magnetic stir bar, and SEVs were added for 30 min prior to antibiotic dosing to allow for climate acclimation. The model was maintained at 35 to 37°C ambient air and fresh medium instilled via a continuous syringe pump system (New Era Pump Systems, Inc.) to provide a human pharmacokinetic simulation of the antibiotics. All model experiments were performed in duplicate flasks to ensure reproducibility with two SEVs collected for each time point ($n = 4$ SEVs per time point). After collection from the model, SEVs were weighed and placed in sterile 1.5-ml microcentrifuge tubes containing 1.0-mm sterile glass beads, and 500 μl of trypsin (from a 25-mg/ml stock) was added (Fig. 4). Tubes were vortexed at medium speed for 1 h, and digested SEVs were plated on MHA plates using a WASP 2 spiral plater (Microbiology International, Frederick, MD). Bacteria were quantified by using a Scan 300 colony counter (Interscience, Woburn, MA), and data are reported as CFU/g of SEV tissue.

Simulated antimicrobial regimens. All regimens were derived from human pharmacokinetic data and standard dosing regimens for humans with MSSA infections previously published and to mimic previous exposures in the rabbit endocarditis model (14) as follows: OXA, 2 g infused every 6 h (10); or CFZ, 2 g infused every 8 h (40, 41). Antibiotics were administered as boluses over 1 min into a Luer lock port of the flask (Fig. 4) at the scheduled administration time over a 72-h dosing duration. The predicted pharmacokinetics of each regimen are provided in Table 2.

Pharmacokinetic analysis and exposure determination. Pharmacokinetic samples were obtained in duplicate through the injection port of each model from 0 to 72 h for verification of target antibiotic concentration attainment. All samples were stored at −80°C until ready for analysis. Concentrations of

OXA were determined by bioassay using *Kocuria rhizophila* ATCC 9341 on MHA as previously described (41). The concentrations of CFZ were determined by bioassay using the test organism *Bacillus subtilis* ATCC 6633 on MHA (42). The half-lives, areas-under-the-curve 0 to 24 h (AUC), and maximum and minimum concentrations (C_{max} and C_{min}) of each antibiotic were determined by the trapezoidal method utilizing Prism (GraphPad Software, Inc.). The observed fAUC/MIC and %fT>MIC values were determined in Prism and reported for OXA and CFZ.

Assessment for emergence of variants with high-level β-lactam resistance in the *ex vivo* model. Samples (100 μl) from each time point were parallel plated onto MHA plates containing either no antibiotic or 4-fold the respective β-lactam initial MICs to assess for the emergence of high-level-resistance mutants. The plates were then examined for growth after 48 h of incubation at 35°C. Specific CFZ or OXA MICs were then determined on selected colonies exhibiting growth on the respective antibiotic-containing agar plates to quantify the actual changes in MIC over the 72-h β-lactam exposure period.

Statistical analysis. Bacterial counts, expressed as the $\log_{10}$ CFU/g, in SEVs at each time point were determined for each antibiotic treatment and growth condition for each strain. The AUBC, defined as area under the bacterial growth curves over the 72-h experiments, was also calculated. A two-way analysis of variance was used with a Tukey's *post hoc* test to compare bacterial counts and an AUBC with a P value of ≤ 0.05 for significance. All statistical comparisons were analyzed using Prism 8 (GraphPad Software, San Diego, CA).

ACKNOWLEDGMENTS

This study was supported in part by research grants from the National Institutes of Health to A.S.B. (NIAID 1RO1AI146078-01) and W.E.R. (NIAID 1RO1AI132627-02).

We thank Sally Griffith-Oh for creating the figure illustration.

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