

# Sensitizing methicillin-resistant *Staphylococcus aureus* (MRSA) to cefuroxime: the synergic effect of bicarbonate and the wall teichoic acid inhibitor ticlopidine

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**ABSTRACT** Methicillin-resistant *Staphylococcus aureus* (MRSA) strains are a major challenge for clinicians due, in part, to their resistance to most  $\beta$ -lactams, the first-line treatment for methicillin-susceptible *S. aureus*. A phenotype termed “NaHCO<sub>3</sub>-responsiveness” has been identified, wherein many clinical MRSA isolates are rendered susceptible to standard-of-care  $\beta$ -lactams in the presence of physiologically relevant concentrations of NaHCO<sub>3</sub>, *in vitro* and *ex vivo*; moreover, such “NaHCO<sub>3</sub>-responsive” isolates can be effectively cleared by  $\beta$ -lactams from target tissues in experimental infective endocarditis (IE). One mechanistic impact of NaHCO<sub>3</sub> exposure on NaHCO<sub>3</sub>-responsive MRSA is to repress WTA synthesis. This NaHCO<sub>3</sub> effect mimics the phenotype of *tarO*-deficient MRSA, including sensitization to the PBP2-targeting  $\beta$ -lactam, cefuroxime (CFX). Herein, we further investigated the impacts of NaHCO<sub>3</sub> exposure on CFX susceptibility in the presence and absence of a WTA synthesis inhibitor, ticlopidine (TCP), in a collection of clinical MRSA isolates from skin and soft tissue infections (SSTI) and bloodstream infections (BSI). NaHCO<sub>3</sub> and/or TCP enhanced susceptibility to CFX *in vitro*, by both minimum inhibitor concentration (MIC) and time-kill assays, as well as in an *ex vivo* simulated endocarditis vegetations (SEV) model, in NaHCO<sub>3</sub>-responsive MRSA. Furthermore, in experimental IE (presumably in the presence of endogenous NaHCO<sub>3</sub>), pre-exposure to TCP prior to infection sensitized the NaHCO<sub>3</sub>-responsive MRSA strain (but not the non-responsive strain) to enhanced clearances by CFX in target tissues. These data support the notion that NaHCO<sub>3</sub> is acting similarly to WTA synthesis inhibitors, and that such inhibitors have potential translational applications in the treatment of certain MRSA strains in conjunction with specific  $\beta$ -lactam agents.

**KEYWORDS** methicillin-resistant *Staphylococcus aureus* (MRSA),  $\beta$ -lactams, NaHCO<sub>3</sub>-responsive, penicillin-binding proteins (PBPs), wall teichoic acid (WTA) synthesis, experimental infective endocarditis (IE)

Methicillin-resistant *Staphylococcus aureus* (MRSA) strains are a major clinical threat due to the large variety of invasive syndromes caused by such strains, as well as their broad antimicrobial resistances (1–3). Recently, however, a novel phenotype has been documented among large collections of clinical MRSA bloodstream infection (BSI) and skin and soft tissue infection (SSTI) isolates, termed “NaHCO<sub>3</sub>-responsiveness”; in this phenotype, many MRSA isolates are rendered “susceptible” *in vitro* to the  $\beta$ -lactams, cefazolin (CFZ) and oxacillin (OXA), in the presence of physiologically relevant concentrations of NaHCO<sub>3</sub> (4–6). The translatability of this *in vitro* phenotype has been established in both *ex vivo* and *in vivo* endocarditis models (4, 7, 8).

Mechanistically, NaHCO<sub>3</sub>-mediated susceptibility to CFZ and OXA appears related to multiple impacts on penicillin-binding protein (PBP) 2a expression and functionality

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(9–11). In addition, NaHCO<sub>3</sub> has been shown to affect the expression and production of components required for PBP2a activity, including the PBP2a chaperone system, *vrSA-prSA*, and wall teichoic acid (WTA) synthesis (9, 12). Of interest, disruption of WTA synthesis, with agents such as ticlopidine (TCP), tarocins, and tunicamycin, is also known to sensitize MRSA to  $\beta$ -lactams, especially those that specifically target PBP2 (13–16). These latter anti-WTA agents share the capacity to inhibit the activity of enzymes involved in early WTA synthesis (13, 15–17).

As mentioned above, we recently demonstrated that NaHCO<sub>3</sub> was capable of altering WTA production in a small cohort of MRSA BSI isolates, and that NaHCO<sub>3</sub> and TCP were similarly capable of sensitizing MRSA to the PBP2-targeting  $\beta$ -lactam, cefuroxime (CFX) (12). There were no further *in vitro* synergistic enhancements of NaHCO<sub>3</sub> plus TCP on CFX sensitization by minimum inhibitory concentration (MIC) assay, suggesting that NaHCO<sub>3</sub> and TCP may have the same biological target.

In the present study, we investigated (i) the ability of NaHCO<sub>3</sub> and TCP to sensitize MRSA to CFX in BSI and SSTI isolates in both static (MIC) and dynamic (time-kill) assays; and (ii) both the *ex vivo* and *in vivo* translatability of *in vitro* susceptibility assays with CFX, NaHCO<sub>3</sub>, and/or TCP, utilizing *ex vivo* simulated endocarditis vegetations (SEV) and rabbit infective endocarditis (IE) models.

## RESULTS

### *In vitro* synergy of TCP, NaHCO<sub>3</sub>, and CFX

Previously, both TCP and NaHCO<sub>3</sub> were each shown to sensitize NaHCO<sub>3</sub>-responsive MRSA BSI isolates to the PBP2-specific inhibitor, CFX (12). In the current cohort, which has been enlarged by the addition of MRSA SSTI isolates, a similar phenotype was observed, wherein NaHCO<sub>3</sub>-responsive MRSA isolates were sensitized to CFX by TCP or NaHCO<sub>3</sub>; in contrast, the susceptibilities of non-responsive strains were not impacted by these agents (Table 1). As observed before, the MICs of CFX were similar for NaHCO<sub>3</sub>-responsive strains exposed to TCP or NaHCO<sub>3</sub> alone, although no additional CFX MIC reductions occurred when TCP and NaHCO<sub>3</sub> were combined.

To further quantify the dynamic impacts of TCP and NaHCO<sub>3</sub> on CFX susceptibility, time-kill assays were used in four prototype strains: two NaHCO<sub>3</sub>-responsive (MRSA

**TABLE 1** Minimum inhibitory concentrations (MICs) of cefuroxime (CFX) for NaHCO<sub>3</sub>-responsive and non-responsive MRSA isolates grown in the presence and absence of TCP and/or NaHCO<sub>3</sub>

	Strain	Isolate source	Cefuroxime MIC ( $\mu$ g/mL)			
			Ca-MHB Tris		Ca-MHB Tris 44 mM NaHCO <sub>3</sub>	
			W/o TCP <sup>a</sup>	+ TCP <sup>b</sup>	W/o TCP	+ TCP
NaHCO <sub>3</sub> -responsive	MRSA 11/11 <sup>c</sup>	BSI <sup>d</sup>	256	8	4	8
	MW2 <sup>c</sup>	BSI	128	8	8	8
	8010	SSTI <sup>d</sup>	256	16	8	16
	8029	SSTI	256	8	8	16
	8058	SSTI	128	16	16	8
	8074	SSTI	256	32	16	32
	8129	SSTI	128	16	8	8
Non-responsive	COL <sup>c</sup>	BSI	>512	>512	512	>512
	BMC1001 <sup>c</sup>	BSI	>512	>512	>512	>512
	8049	SSTI	>256	>256	>256	>256
	8080	SSTI	>256	>256	>256	>256
	8086	SSTI	32	32	32	16
	8096	SSTI	>256	>256	>256	>256
	8131	SSTI	16	16	8	16

<sup>a</sup>TCP = ticlopidine.

<sup>b</sup>TCP exposure is 32  $\mu$ g/mL in all conditions.

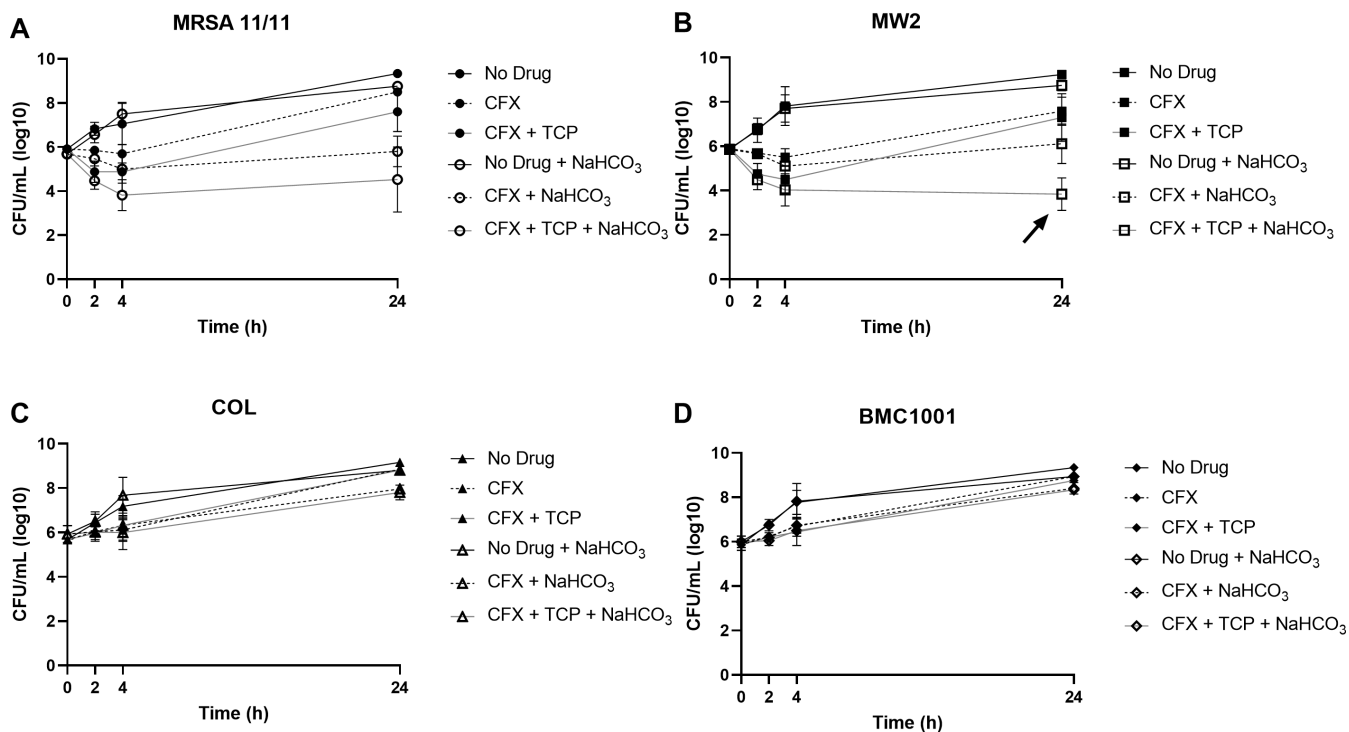
<sup>c</sup>Data previously published in Ersoy et al. (12).

<sup>d</sup>BSI, bloodstream infection; SSTI, skin and soft tissue infection.

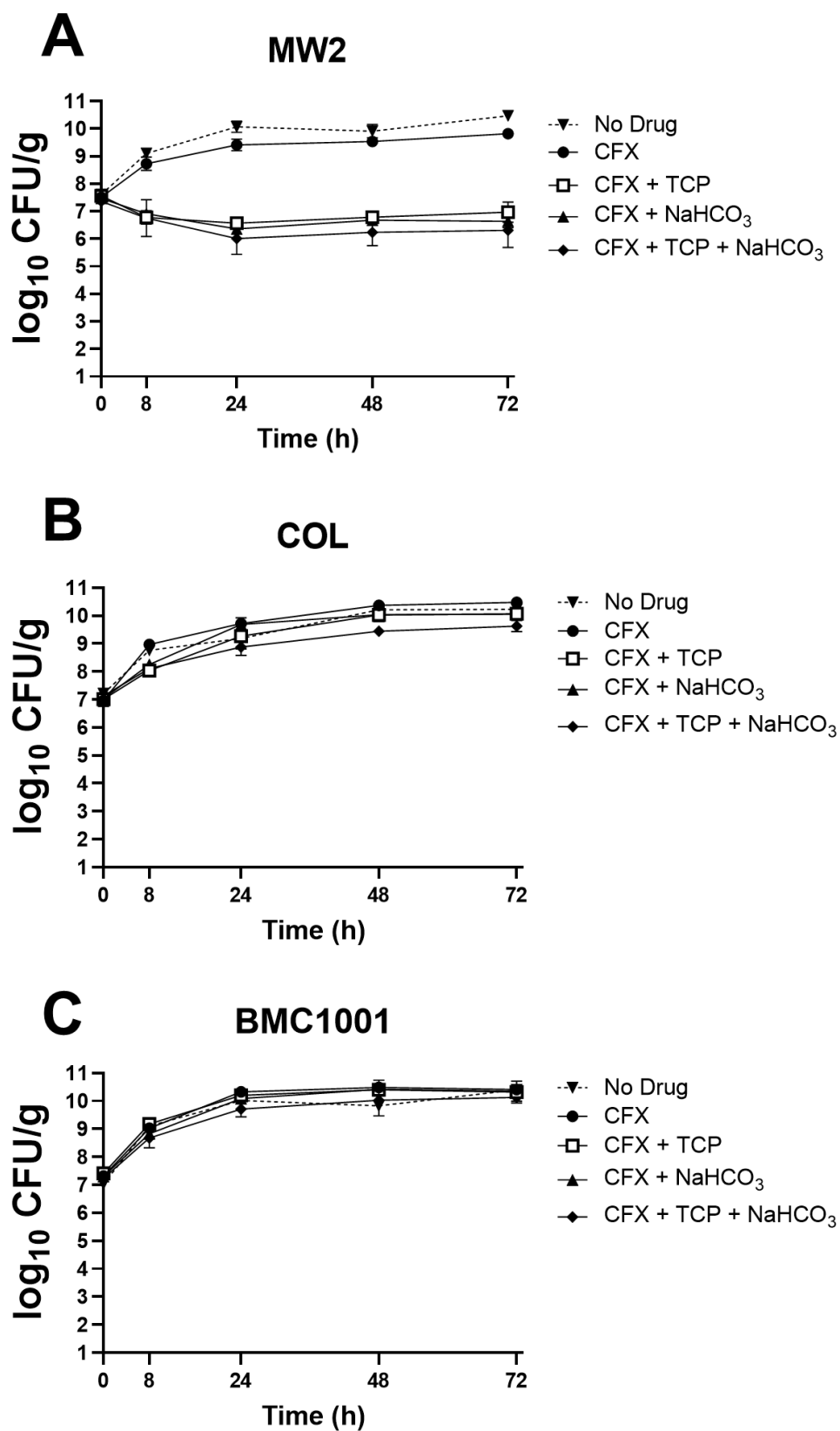
11/11 and MW2) and two NaHCO<sub>3</sub>-non-responsive (COL and BMC1001). Although the MICs of CFX were similar in the presence of TCP or NaHCO<sub>3</sub> alone or in combination, the combination of TCP plus NaHCO<sub>3</sub> resulted in several logs of increased killing by CFX in NaHCO<sub>3</sub>-responsive strains at 24 h vs each agent alone, CFX and NaHCO<sub>3</sub> in combination, or TCP and CFX in combination (Fig. 1A and B). This triple combination exerted “synergy” for strain MW2 (>2 log<sub>10</sub> CFU/mL reductions,  $P < 0.0001$  CFX + TCP + NaHCO<sub>3</sub> vs both CFX + TCP or CFX + NaHCO<sub>3</sub> at 24-h exposures) (Fig. 1B). This suggested the presence of synergistic bactericidal effects of CFX plus TCP plus NaHCO<sub>3</sub> in combination that were not observed in more static MIC assays. As expected, TCP and NaHCO<sub>3</sub>, alone or in combination, did not enhance bacterial killing by CFX in non-responsive strains (Fig. 1C and D).

### Ex vivo CFX synergy with NaHCO<sub>3</sub> and TCP in the SEV model

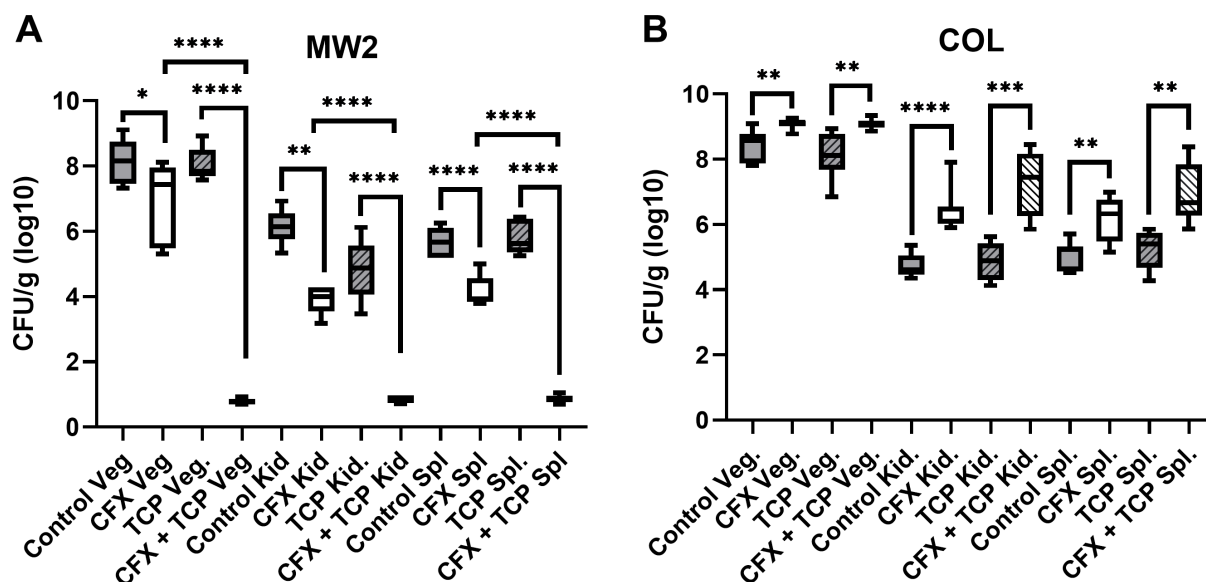
This SEV model mimics two key microenvironments that MRSA strains are exposed to *in vivo*: (i) high-inoculum growth within cardiac vegetations; and (ii) human-equivalent pharmacokinetic-pharmacodynamic (PK-PD) dosing regimens. Thus, this model is widely considered as a “bridge” between *in vitro* and *in vivo* studies (18, 19). SEV time-kill assays for the NaHCO<sub>3</sub>-responsive strain, MW2, demonstrated (i) no difference in 72-h SEV growth curves between exposures to chamber fluid media alone (CA-MHB Tris), CFX alone or media supplemented with NaHCO<sub>3</sub> or TCP alone (growth curves for supplementation with NaHCO<sub>3</sub> or TCP alone not shown); and (ii) a >3 log<sub>10</sub> CFU/g reduction for CFX supplemented media with TCP, NaHCO<sub>3</sub>, or both, indicating synergy between CFX combined with TCP and/or NaHCO<sub>3</sub> (Fig. 2A), as predicted by *in vitro* MIC studies (Table 1). Neither combination regimen exerted a bactericidal effect over the 72-h exposure period. In contrast, for the non-responsive strains, COL or BMC1001, in the SEV model, neither TCP nor NaHCO<sub>3</sub> enhanced killing of CFX, as expected from *in vitro* studies (Fig. 2B and C; Table 1).



**FIG 1** Time-kill curves for NaHCO<sub>3</sub>-responsive strains MRSA 11/11 (A) and MW2 (B), and non-responsive strains COL (C) and BMC1001 (D) exposed to CFX, with or without NaHCO<sub>3</sub> and TCP. Cells were incubated without drug for 3 h to enter log phase. For all assays, drug concentrations are 30 μg/mL CFX and 32 μg/mL TCP.



**FIG 2** SEV kill curves for NaHCO<sub>3</sub>-responsive strain MW2 (A), and non-responsive strains COL (B) and BMC1001 (C) exposed to CFX, with or without NaHCO<sub>3</sub> and TCP. CFX dosing mimics human PK/PD, while TCP dosing was optimized to reach a steady state of 8 μg/mL throughout the experiment.



**FIG 3** Tissue burdens (CFU/g) of NaHCO<sub>3</sub>-responsive strain MW2 (A) and non-responsive strain COL (B) following infection in a rabbit model of IE. Rabbits were grouped as follows: control = infected with MW2 or COL and untreated; CFX = infected with MW2 or COL and treated with CFX; TCP = infected with MW2 or COL that were exposed to TCP prior to infection and untreated; TCP + CFX = infected with MW2 or COL that were exposed to TCP prior to infection and treated with CFX. Veg. = cardiac vegetation, Kid. = kidney, Spl. = spleen. Statistics were calculated by an unpaired Student's *t*-test, \**P* < 0.05, \*\**P* < 0.01, \*\*\**P* < 0.001, \*\*\*\**P* < 0.0001.

### CFX synergy with endogenous NaHCO<sub>3</sub> and/or TCP pre-exposures in experimental IE

To investigate the ability of TCP to sensitize MRSA to CFX in the presence of endogenous HCO<sub>3</sub><sup>-</sup>, a rabbit model of experimental IE was used. MRSA cells were pre-exposed to TCP prior to infection to “sensitize” them for potential CFX synergy *in vivo*. Since TCP has potent anti-platelet aggregation activity and the pathogenesis of IE is highly platelet-dependent (15, 20–22), this pre-exposure strategy was employed rather than direct TCP treatments of animals. Furthermore, TCP is rapidly and extensively metabolized in the liver (23). As predicted by MIC, time-kill, and SEV data above, CFX treatment of rabbits infected with the “un-sensitized” NaHCO<sub>3</sub>-responsive strain, MW2, resulted in modest but significant reductions (0.5 to 2 log<sub>10</sub> CFU/g reductions) in MRSA bio-burdens in all target tissues (Fig. 3A). Of note, when MW2 was “sensitized” by TCP exposure prior to infection, CFX treatment resulted in significantly more MRSA killing (4 to 7 log<sub>10</sub> CFU/g reductions) than infection with un-sensitized cells, to near sterilization (detection-limit) levels in all three target tissues (Fig. 3A). As expected, CFX treatment of rabbits infected with the non-responsive strain COL, with or without TCP priming, did not result in any reductions of target tissue MRSA bio-burdens (Fig. 3B).

### DISCUSSION

The ability to “re-purpose” antibiotics with broad efficacy, which are relatively inexpensive and have well-known modes of action and favorable safety profiles, would be a great benefit to physicians in treating complicated MRSA infections (24, 25). Current treatment options for MRSA infections are more limited, and tend to be less effective and more toxic than standard-of-care β-lactams used for the treatment of methicillin-susceptible *S. aureus* (MSSA) (26–28).

In the current study, we demonstrated five major findings: (i) BSI and SSTI NaHCO<sub>3</sub>-responsive (but not NaHCO<sub>3</sub>-non-responsive) MRSA isolates were sensitized to growth inhibition and killing by the widely used, broad-spectrum, second-generation cephalosporin, CFX (29), in the presence of NaHCO<sub>3</sub> or TCP both *in vitro* and *ex vivo*; (ii) in

growth-inhibitory MIC assays, the reductions in baseline CFX MICs, when combined with either  $\text{NaHCO}_3$  or TCP alone, were not synergistically enhanced by combining all three agents (suggesting that  $\text{NaHCO}_3$  and TCP may have the same biological target); (iii) in contrast, in time-kill assays, the combination of TCP plus  $\text{NaHCO}_3$  did, indeed, enhance the activity of CFX against  $\text{NaHCO}_3$ -responsive MRSA, as compared to CFX plus either TCP or  $\text{NaHCO}_3$ ; (iv) in a SEV model, exposure to CFX with TCP and/or  $\text{NaHCO}_3$  resulted in similar levels of synergistic growth inhibition compared to CFX exposure alone in  $\text{NaHCO}_3$ -responsive MRSA; and (v) TCP pre-exposures sensitized  $\text{NaHCO}_3$ -responsive MRSA to CFX-mediated target tissue MRSA clearances, in the presence of endogenous  $\text{HCO}_3^-$  in experimental IE (i.e.,  $\text{HCO}_3^-$  naturally existing in blood and tissues, typically ranging from ~22 to 45 mM [4, 30]).

Of note, there were discrepancies between the various *in vitro*, *ex vivo*, and *in vivo* assays in determining whether combining CFX with both TCP and  $\text{NaHCO}_3$  resulted in enhanced growth inhibition compared to exposure to CFX with either TCP or  $\text{NaHCO}_3$  alone. Although the MIC and SEV assays did not demonstrate enhanced synergy with the triple combination (CFX + TCP +  $\text{NaHCO}_3$ ), such enhanced efficacy was observed by time-kill assay and pre-exposure to TCP in the experimental rabbit IE model, in  $\text{NaHCO}_3$ -responsive MRSA. We attribute these discrepancies to several factors, including (i) differences in the “metabolic state” of the organism in MIC and SEV vs time-kill and *in vivo* assays, making MRSA in the latter conditions more “susceptible” to such synergy; and/or (ii) the increased production of the inhibitory target (WTA) when initially exposed to TCP and CFX during exponential-phase growth (time-kill assays). Regarding the differences in triple combination synergy in the *ex vivo* vs *in vivo* model, the latter used TCP priming, which appears to further sensitize  $\text{NaHCO}_3$ -responsive MRSA to the effects of CFX. Furthermore, exposure to WTA synthesis-inhibiting molecules has been shown to directly reduce MRSA virulence (31), which may explain the enhanced clearance of TCP pre-exposed  $\text{NaHCO}_3$ -responsive MRSA by CFX in the experimental IE model.

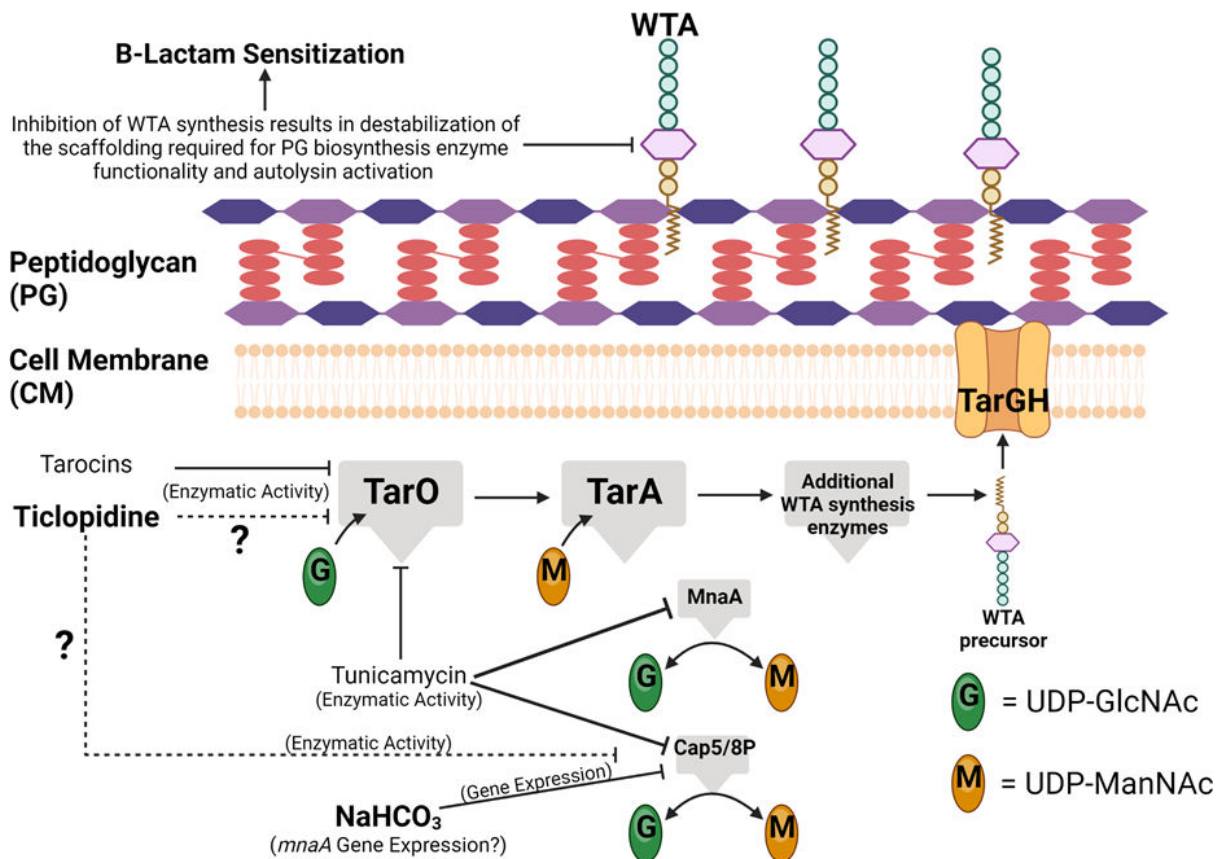
Antibiotic susceptibility is strongly tied to the growth state of the organism, which typically exhibits higher levels of “resistance” to antimicrobials during non-planktonic growth in a biofilm (vs planktonic growth in standard *in vitro* growth media) (32). These differences can confound *in vitro*-determined levels of antibiotic resistance, which typically measures resistance in a planktonic state, against those exhibited during biofilm growth as found in selected host infections. Importantly, the studies presented herein demonstrate that  $\text{NaHCO}_3$  and TCP sensitize MRSA to CFX in both *in vitro*, planktonic growth states (MIC and time-kill assays), as well as in the *ex vivo* SEV model, in which MRSA cells are growing deeply within platelet-fibrin clots, in a biofilm-like state similar to what is observed during infection within a human endovascular lesion in endocarditis (8, 33). These similar sensitization outcomes observed in both these models, in which MRSA growth states differ greatly, further support the notion that both  $\text{NaHCO}_3$  and TCP would be capable of sensitizing certain MRSA strains (i.e.,  $\text{NaHCO}_3$ -responsive strains) to CFX during actual human infection. Finally, the *in vivo* verification of the efficacy of CFX in TCP-pretreated  $\text{NaHCO}_3$ -responsive strains (in the presence of endogenously derived  $\text{NaHCO}_3$ ) in clearing MRSA from human-like infected cardiac vegetations fully underscores the above concepts.

The WTA biosynthetic pathway is a promising target for new antimicrobials, as WTAs are involved in a wide range of key physiological functions including bacterial: host interactions, bacterial virulence, biofilm formation, and peptidoglycan functionality, including cell division and autolysin activity (34). Additionally, WTAs are essential for the cooperative action of PBP2 and PBP4 that results in highly cross-linked peptidoglycan. Both impairment of the first step of WTA synthesis, mediated by the transferase, TarO, and deletion of *tarO* result in sensitization of MRSA to many  $\beta$ -lactams, including those specific for PBP2, such as CFX (15). Exposure to  $\text{NaHCO}_3$  simulates  $\Delta tarO$ -deletion phenotypes in  $\text{NaHCO}_3$ -responsive strains; however,  $\text{NaHCO}_3$  has no direct impact on *tarO* gene expression, either transcriptionally or translationally (12). Thus,  $\text{NaHCO}_3$

appears to impact TarO post-translationally and reduces WTA production exclusively in NaHCO<sub>3</sub>-responsive MRSA (12).

The precise anti-WTA mechanisms of TCP are not totally defined; however, like NaHCO<sub>3</sub>, TCP most likely targets TarO and synergizes with CFX against MRSA (15, 17). It was previously reported that TCP inhibits the enzymatic activity of TarO (15); however, it remains unclear if TCP directly binds to TarO or whether it inhibits the enzymatic activity of TarO via secondary pathways (17). One potential secondary target of TCP and NaHCO<sub>3</sub> is Cap5P, the 2'-epimerase that interconverts UDP-GlcNAc and UDP-ManNAc, the substrates of TarO and another early WTA synthesis step, mediated by TarA (16, 35). Inhibition of UDP-GlcNAc and UDP-ManNAc interconversion has been shown to inhibit WTA synthesis and sensitize MRSA to β-lactams (16). Interestingly, NaHCO<sub>3</sub> was previously demonstrated to inhibit the expression of *cap5/8P* specifically in NaHCO<sub>3</sub>-responsive strains (36), and certain *cap5P* genotypes were specific to non-responsive MRSA strains in a collection of SSTI isolates (6). Although speculative, these latter data provide one potential mechanism in which NaHCO<sub>3</sub> and TCP may be inhibiting the expression and/or function of *cap5P*/Cap5P, thereby, repressing the enzymatic activity of TarO and disrupting WTA synthesis specifically in NaHCO<sub>3</sub>-responsive MRSA. A schematic illustrating this hypothesis is provided in Fig. 4.

Deletion of *tarO* or exposure to targocil (another WTA synthesis-inhibiting agent that targets the translocator, TarG) alters the expression of a similar cadre of virulence and peptidoglycan biosynthesis genes as observed with exposure to NaHCO<sub>3</sub> in NaHCO<sub>3</sub>-responsive MRSA, including *pbp2*, *fntA*, *vraX*, *sasD*, *fntA*, and *fntB* (14, 36, 37). This



**FIG 4** Schematic of potential impacts of TCP and NaHCO<sub>3</sub> on WTA biosynthesis. Previous studies have demonstrated that TCP inhibits WTA biosynthesis (similarly to tarocins and tunicamycin), potentially via impacts on TarO functionality (13, 15, 17). NaHCO<sub>3</sub> has been demonstrated to specifically inhibit the expression of *cap5/8P* in a NaHCO<sub>3</sub>-responsive MRSA strain (36). Inhibition of *cap5/8P* expression, coupled with inhibition of TarO enzymatic activity (via direct or indirect mechanisms), could result in disruption of WTA biosynthesis, as previously observed (12), sensitizing NaHCO<sub>3</sub>-responsive MRSA to β-lactams. Created with BioRender.com.

provides yet another line of evidence that  $\text{NaHCO}_3$  may well impact multiple steps in the WTA biosynthetic pathway. Assessing the ability of  $\text{NaHCO}_3$  and/or TCP to further sensitize a  $\Delta tarO$   $\text{NaHCO}_3$ -responsive MRSA strain to CFX may reveal whether these compounds do, in fact, target TarO and/or act on another aspect of WTA synthesis. In addition, studies utilizing the TarG inhibitor, L-638, in combination with either  $\text{NaHCO}_3$  or TCP may help elucidate their combined impacts on early WTA synthesis. Inhibition of TarG by L-638 has been shown to inhibit cell growth, and suppression of this activity can be achieved by concomitant exposure to early WTA synthesis inhibitors (17).

Other known early WTA synthesis inhibitors, such as tunicamycin and tarocins A and B, have also been shown to sensitize various MRSA strains (including the  $\text{NaHCO}_3$ -non-responsive strain, COL) to a number of  $\beta$ -lactams, perhaps indicating a more potent or broader spectrum of anti-WTA activity than seen with TCP and/or  $\text{NaHCO}_3$  (13, 17). Furthermore, tunicamycin and tarocins showed higher *in vitro* inhibitory activity against TarO than TCP, and although both drug classes act through different mechanisms on WTA synthesis, their synergic effect with  $\beta$ -lactams was demonstrated to be more potent than the effect of TCP (17). As only a relatively modest synergistic effect was observed between  $\text{NaHCO}_3$  and/or TCP with CFX *in vitro* and *ex vivo* in the present study, it would be of interest to determine whether a more robust level of synergy might be observed between  $\text{NaHCO}_3$ ,  $\beta$ -lactams, and either tunicamycin or tarocins *in vitro* and *ex vivo*. Indeed, the significant synergy demonstrated with TCP “sensitizing” used in the *in vivo* IE model indicates a potential role of sequential inhibition of this pathway for maximal anti-MRSA effects. Furthermore, *in vivo* studies in a rabbit IE model, similar to the present studies, utilizing selected  $\beta$ -lactams and tunicamycin or tarocins for the treatment of  $\text{NaHCO}_3$ -responsive and non-responsive MRSA would be very informative. Such investigations are currently in progress in our laboratories.

One limitation of this work is the lack of consensus on the specific target/mechanism of action of TCP. Although some research suggests a direct impact on the enzymatic activity of TarO (15), other studies suggest that TarO is not the specific target of this molecule (17). Further supporting the notion that TCP does not directly affect TarO is its specificity for  $\text{NaHCO}_3$ -responsive MRSA, a strain selectivity not observed with other TarO inhibitors (13, 17). As proposed above, it is possible that TCP also targets the 2'-epimerase Cap5P, a known target of  $\text{NaHCO}_3$  in  $\text{NaHCO}_3$ -responsive strains, similar to the ability of tunicamycin to target the 2'-epimerase MnaA (16). Additional investigations are needed to identify the specific targets of both  $\text{NaHCO}_3$  and TCP, as they relate to WTA synthesis, and the mechanism of their ability to specifically sensitize  $\text{NaHCO}_3$ -responsive MRSA strains to  $\beta$ -lactams.

Overall, our studies herein provide evidence of the translatability of the  $\text{NaHCO}_3$ -responsive phenotype as it relates to the sensitization of MRSA to selected  $\beta$ -lactams *in vivo*.  $\text{NaHCO}_3$  appears to be sensitizing MRSA to  $\beta$ -lactams, in part, via impacts on WTA synthesis. Future development of inhibitors of the WTA pathway holds a promise to “rescue”  $\beta$ -lactam effectiveness for clinical MRSA treatment.

## MATERIALS AND METHODS

### Bacterial isolates and growth conditions

The MRSA isolates utilized in this study are clinical BSI or SSTI isolates that have been previously described and characterized for their  $\text{NaHCO}_3$ -responsive phenotypes (4, 6). Isolates were stored at  $-80^\circ\text{C}$  until thawed for use and isolated on tryptic soy agar (TSA). For all *in vitro* susceptibility assays, cells were grown in cation-adjusted Mueller Hinton Broth (BD, Difco) with 100 mM Tris buffer (CA-MHB Tris, pH  $7.3 \pm 0.1$ )  $\pm$  44 mM  $\text{NaHCO}_3$  (CA-MHB Tris, 44 mM  $\text{NaHCO}_3$ ) at  $37^\circ\text{C}$ . For *in vivo* studies, cells were grown overnight at  $37^\circ\text{C}$  in Brain Heart Infusion (BHI, BBL) with or without 32  $\mu\text{g}/\text{mL}$  TCP, as this dose of TCP provided the best synergy with CFX in *in vitro* susceptibility assays. Exposure (“sensitizing”) to TCP did not impact overnight cell density prior to animal infection (Fig. S1).



## MIC and quantitative time-kill assays

MIC and time-kill assays were carried out as previously described (4). Briefly, for MIC assays, cells were grown overnight in the indicated growth medium, then diluted to  $5 \times 10^5$  CFU/mL into the same medium containing twofold serial dilutions of CFX, with or without 32  $\mu\text{g/mL}$  TCP, on 96-well plates, as per Clinical and Laboratory Standards Institute guidelines (38, 39). Plates were incubated overnight at 37°C, and the MIC was scored at the first well in which turbidity was visually reduced. For time-kill assays, cells were grown overnight in the indicated growth medium, then diluted to  $5 \times 10^5$  CFU/mL in the same growth medium on 96-well plates and incubated at 37°C for 3 h to reach log phase. After reaching the log phase, the cells were diluted back to  $5 \times 10^5$  CFU/mL into the same growth medium in the presence or absence of 30  $\mu\text{g/mL}$  CFX and 32  $\mu\text{g/mL}$  TCP. This TCP concentration was selected following extensive piloting studies revealing this to be the optimal concentration to disclose synergy between TCP and CFX *in vitro*. Viable cells were quantified by plating on TSA at 0, 2, 4, and 24 h following incubation with drug.

## Ex vivo PK-PD SEV model

Antibiotic PK-PD simulations were performed with *ex vivo* SEVs prepared as previously described (8). SEVs contain 3–3.5 g/dL of albumin and 6.8–7.4 g/dL of total protein (equating to human physiologic levels) and weigh ~0.7 g. The central (“fluid”) compartment model for the SEVs consists of a 150-mL flask glass, maintained at 35–37°C ambient air and fresh media (MHB or MHB + 44 mM  $\text{NaHCO}_3$ ) instilled via a continuous syringe pump system (New Era Pump Systems, Inc.). The models were performed in duplicate flasks to ensure reproducibility with two SEVs collected for each time point ( $n = 4$  SEVs per time point). Simulated human-equivalent antibiotic regimens used for severe infections were derived from human PK data as follows: CFX 750 mg every 8 h, infused over 2 min ( $C_{\text{max}}$  50  $\mu\text{g/mL}$ ,  $C_{\text{min}}$  0.72  $\mu\text{g/mL}$ ,  $T_{1/2}$  1.2 h). Standard TCP regimens of 250 mg twice daily in humans display nonlinear pharmacokinetics, with a terminal half-life of repeated dosing of 4–5 d. Therefore, we simulated steady-state concentrations during the terminal half-life (8  $\mu\text{g/mL}$ ) (40) by including TCP 8  $\mu\text{g/mL}$  in the model and supplemental media over a 72-h duration to achieve this constant TCP exposure while simulating the CFX elimination rate constant ( $k_e = 0.53 \text{ h}^{-1}$ ). Model SEVs were sampled and processed for CFU enumeration as previously described (8).

## Rabbit model of MRSA IE

To quantify the synergistic impacts of TCP and endogenous  $\text{HCO}_3^-$  on CFX activity in  $\text{NaHCO}_3$ -responsive vs non-responsive strains, a well-characterized rabbit model of indwelling catheter-induced aortic valve IE was used (41). Rabbits were infected intravenously at 48 h after catheter placement with  $2 \times 10^5$  CFU/animal of the indicated strain; this inoculum represents the  $\text{ID}_{95}$  for inducing IE as established by extensive pilot experiments for each strain (data not shown). At 24-h post-infection, the animals were randomized into either untreated control groups (sacrificed at this time-point as a therapeutic baseline) or CFX-treated groups (100 mg/kg administered by intramuscular injection, tid for 4 d). This CFX dosing regimen was based on previously established CFX dosing strategies in MSSA rabbit IE models (4, 42, 43).

For CFX-treated animals, at 24 h after the last CFX treatment (to circumvent any antibiotic carryover effects), animals were sacrificed, and their cardiac vegetations, kidneys, and spleen were aseptically removed and quantitatively cultured on TSA plates as previously described (44). For untreated controls, animals were similarly sacrificed at 24-h post-infection to establish a baseline target tissue MRSA bio-burden to compare to CFX-treated animals. Counts were expressed as mean  $\log_{10}$  CFU per gram of tissue ( $\pm$  SD). The limit of detection in target organ cultures in this model, based on average target tissue weights, is  $\leq 2 \log_{10}$  CFU/g. All culture-negative vegetations, although designated as “sterile,” were still assigned a limit-of-detection  $\log_{10}$  CFU/g designation based on

their weights, for statistical purposes, to enable comparisons between groups. This is a standard procedure used in this model.

## Statistical analyses

An unpaired Student's *t*-test was used to make all statistical comparisons, with  $*P < 0.05$ ,  $**P < 0.01$ ,  $***P < 0.001$ , and  $****P < 0.0001$ . For *in vitro* kill curves, statistics were calculated on the viable cell means at 24 h for each group.

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## ADDITIONAL FILES

The following material is available [online](#).

## Supplemental Material

**Figure S1 (AAC01627-23-s0001.jpg).** Impact of ticlopidine pre-exposure on overnight cell density.

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