Received: 9 March 2018 | Accepted: 16 June 2018

DOI: 10.1111/cbdd.13361

RESEARCH ARTICLE

WILEY

Tricyclic amine antidepressants suppress β-lactam resistance in methicillin-resistant *Staphylococcus aureus* (MRSA) by repressing mRNA levels of key resistance genes

Kyra Gillard | **Heather B. Miller** | **Meghan S. Blackledge**

Department of Chemistry, High Point University, High Point, North Carolina

Correspondence
Heather B. Miller and Meghan S. Blackledge, One University Parkway, High Point, NC 27268.
Emails: hmiller@highpoint.edu; mblackle@highpoint.edu

Funding information
High Point University (High Point, NC)

Abstract

Methicillin-resistant *Staphylococcus aureus* (MRSA) is the leading cause of recurrent infections in humans including endocarditis, pneumonia, and toxic shock syndrome. Novel therapeutics to treat MRSA and other resistant bacteria are urgently needed. Adjuvant therapy, which uses a non-toxic compound to repotentiate the toxic effects of an existing antibiotic, is an attractive response to the growing resistance crisis. Herein, we describe the evaluation of structurally related, FDA-approved tricyclic amine antidepressants that selectively repotentiate MRSA to β-lactam antibiotics. Our results identify important structural features of the tricyclic amine class for β-lactam adjuvant activity. Furthermore, we describe the mechanism of action for our lead compound, amoxapine, and illustrate that it represses the mRNA levels of key β-lactam resistance genes in response to β-lactam treatment. This work is novel in that it highlights an important class of small molecules with the ability to simultaneously inhibit production of both β-lactamase and penicillin binding protein 2a.

KEYWORDS

β-lactam resistance, adjuvant, *blaZ*, *mecA*, methicillin-resistant *Staphylococcus aureus*, tricyclic amine antidepressant

1 | INTRODUCTION

Antibiotic resistance has become the most pressing health crisis of the 21st century,[1–4] prompting officials in both the United States and around the world to call for action.[5,6] While antibiotic-resistant infections were limited to healthcare and hospital settings for several decades, recently, community-acquired drug-resistant infections have become increasingly common.[7] Resistance has been observed to every known class of antibiotic,[8] rendering our current arsenal of therapeutics increasingly useless.

Methicillin-resistant *Staphylococcus aureus* (MRSA) is an opportunistic pathogen associated with soft tissue and systemic infections in humans.[9] In 2005 alone, deaths from MRSA outnumbered those from AIDS, Parkinson's disease, emphysema, and homicide, combined.[10] Although rigorous healthcare initiatives have lowered infection rates by nearly 50% in the last decade, MRSA remains a prevalent and deadly pathogen in both community and healthcare settings.[11] Hospital-acquired MRSA (HA-MRSA) infections traditionally exhibit multidrug resistance and lead to increased length of hospitalization, higher treatment costs to patients, and higher mortality rates.[10]

Bacteria have evolved numerous methods for evading destruction by antibiotics. Resistant strains, including MRSA, have developed genetically encoded resistance mechanisms to ensure their survival. The most common genotypic resistance

Abbreviations: MIC, minimum inhibitory concentration; MRSA, methicillin-resistant *Staphylococcus aureus*; MSSA, methicillin-sensitive *Staphylococcus aureus*; RT-qPCR, reverse time-quantitative polymerase chain reaction.

mechanisms fall into one of the three broad classes: (a) lowered intracellular antibiotic accumulation by decreased antibiotic uptake or increased efflux, (b) target modification to decrease the affinity of the target for the antibiotic, and (c) antibiotic inactivation via chemical modification or degradation of the antibiotic.[12] In addition to the proteins directly involved in these resistance mechanisms, bacteria possess numerous proteins responsible for activating and regulating these mechanisms. These regulatory networks allow the bacteria to detect the presence of an antibiotic and initiate a signal cascade that results in either activation or upregulation of proteins necessary for resistance.

β-lactam antibiotics are mechanism-based inactivators of penicillin binding protein 2 (PBP2), a transpeptidase responsible for bacterial cell wall biosynthesis. When bacterial cell wall biosynthesis is inhibited, cell growth halts and the bacterial cell eventually dies. MRSA uses extensive and overlapping regulatory networks to sense and respond to β-lactam antibiotics. Acylation of the sensor proteins BlaR1 and MecR1 initiates a signaling cascade leading to degradation of the transcriptional repressors BlaI and MecI and production of β-lactamase and penicillin binding protein 2a (PBP2a), respectively (Figure 1). β-lactamase, encoded by the gene *blaZ*, inactivates β-lactam antibiotics via hydrolysis, thereby preventing the antibiotics from disrupting cell wall biosynthesis. PBP2a, encoded by the gene *mecA*, is produced as a target modification; it maintains transpeptidase activity like PBP, but has reduced affinity toward many β-lactam antibiotics. Thus, production of PBP2a allows continued bacterial cell wall biosynthesis in the presence of certain β-lactam antibiotics.

Clearly, novel therapeutics to treat MRSA and other resistant bacteria are urgently needed. Unfortunately, the rate of antibiotic discovery has slowed considerably in the last 30 years due to lack of novel drug targets and scaffolds and the poor return on investment for pharmaceutical companies.[8] Furthermore, bacteria develop resistance to new antimicrobials almost as soon as we discover them. Adjuvant therapy, which uses a non-toxic compound to repotentiate the toxic effects of an existing antibiotic, is an attractive response to the many pitfalls of antibiotic discovery programs. Adjuvants alone are non-toxic, so bacteria have very little selective pressure to develop resistance to these molecules, which increases their therapeutic lifetime.[13] Additionally, adjuvants restore the therapeutic efficacy of existing antibiotics whose targets, mechanisms of action, and dosing regimens have been described fully. Antibiotic adjuvants have already shown efficacy in clinical settings. Clavulanic acid, a β-lactam with little antibiotic activity, has been successfully used in combination with amoxicillin as a broad-spectrum antibiotic treatment for over three decades. Marketed as Augmentin® (GSK), it was the best-selling antibiotic in 2001, illustrating the therapeutic and economic attractiveness of effective combination therapies.[14]

In an effort to identify novel antibiotic adjuvants, we screened a number of FDA-approved compounds for the

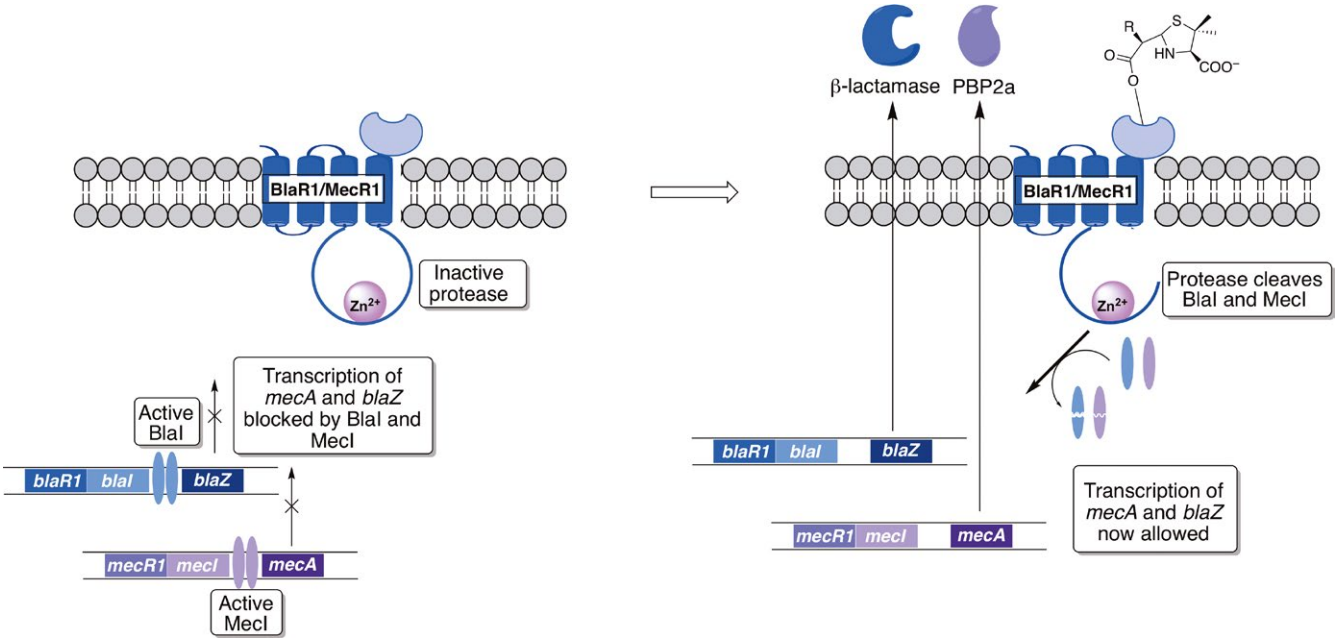

FIGURE 1 β-lactam resistance in MRSA is mediated by the *bla* and *mec* operons. When not under threat by β-lactam antibiotics (left), the transcriptional repressors BlaI and MecI exist as dimers and block transcription of their respective operons. Upon exposure to β-lactam antibiotics (right), the periplasmic domains of BlaR1 and MecR1 are acylated by the antibiotics, causing a conformational change that activates the zinc protease activity in the cytoplasmic domain. The zinc protease cleaves BlaI or MecI, thus allowing the corresponding operon to be transcribed and leads to production of β-lactamase (encoded by *blaZ*) and PBP2a (encoded by *mecA*)

ability to potentiate oxacillin in MRSA.[15] Amoxapine, a tricyclic amine antidepressant with a dibenzoxazepine core, was identified in this screen (Figure 2). As amoxapine is one component of a larger structural class of molecules that has been extensively studied, we asked whether other structurally related compounds displayed similar antibiotic adjuvant activities against MRSA and sought to elucidate their mechanism of antibiotic repotentiation. Herein, we report the generation of a focused structure–activity relationship (SAR) between the tricyclic amine antidepressants and β-lactam adjuvant activity. We further illustrate that these molecules selectively and simultaneously modulate the mRNA levels of *blaZ* and *mecA,* thereby disarming MRSA's chief resistance mechanisms and restoring the therapeutic utility of β-lactam antibiotics.

2 | METHODS AND MATERIALS

2.1 | Bacterial strains

Methicillin-sensitive *S. aureus* (MSSA) (ATCC 29213) and methicillin-resistant *S. aureus* (MRSA) (ATCC 43300, ATCC 33591) were purchased from the American Type Culture Collection (ATCC). Methicillin-resistant *S. aureus* strain USA300 was purchased from BEI Resources.

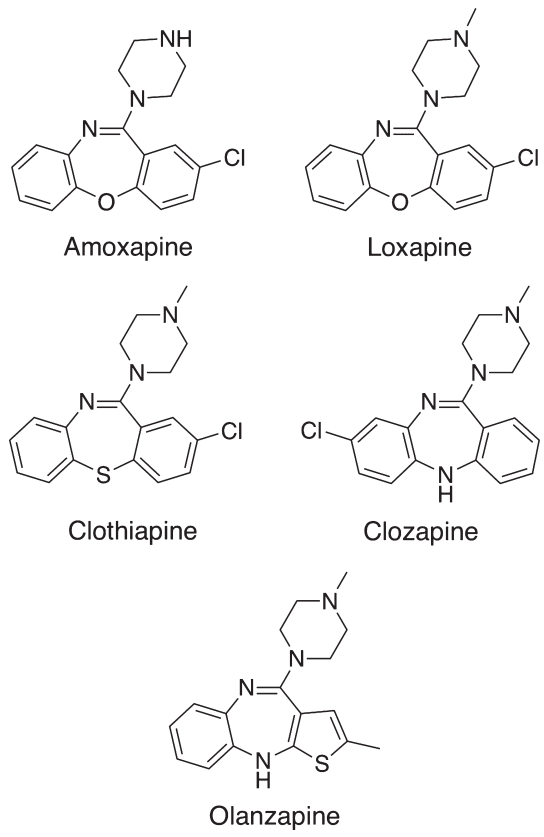

FIGURE 2 Tricyclic amine antidepressants investigated in this study

2.2 | Chemicals and reagents

Cation-adjusted Mueller–Hinton Broth II (CAMHB) and tryptic soy broth (TSB) were purchased from BD. Biological grade DMSO and defibrinated sheep blood was purchased from VWR. Amoxapine, clozapine, loxapine, clothiapine, and olanzapine were purchased from TCI America. Nitrocefin was purchased from Sigma-Aldrich. Molecular Probes Live/Dead BacLight assay reagent was purchased from ThermoFisher. RT-qPCR reagents were purchased from BioRad. PCR primers used in this study were purchased through IDT and are listed in Supporting Information.

2.3 | Minimum inhibitory concentration

Minimum inhibitory concentrations were determined using a standard serial broth microdilution method according to CLSI guidelines.[16] Mid-log phase cultures were diluted to a concentration of approximately 5×10^5 CFU/ml in CAMHB. Wells 2–11 of a 96-well polyvinylchloride microtiter plate were inoculated with 100 µl of bacterial suspension. One hundred microliters of uninoculated CAMHB were added to well 12 to serve as a negative control. The top wells were inoculated with 200 µl of bacterial suspension with either antibiotics or compounds added. Serial dilutions were performed in wells 2–10, leaving well 11 to serve as the positive control. The microtiter plates were covered with Glad Press-n-Seal and incubated at 37°C for 18 hr without agitation. Plates were scored by visual detection of well turbidity. Minimum inhibitory concentrations were recorded as the lowest concentration of compound or antibiotic at which no visible bacterial growth was observed with the unaided eye.

2.4 | Antibiotic repotentiation

MRSA (ATCC 43300, ATCC 33591, USA300) was grown overnight in CAMHB at 37°C with shaking. The overnight culture was diluted into fresh CAMHB to a concentration of 5×10^5 CFU/ml. The cell suspension (3 ml) was aliquoted into sterile culture tubes and compound was added to the appropriate concentration (≤25% of the compound MIC). Wells 2–11 of a 96-well polyvinylchloride microtiter plate were inoculated with 100 µl of bacterial suspension with added compound. One hundred microliters of uninoculated CAMHB were added to well 12 to serve as a negative control. The top wells were inoculated with 200 µl of bacterial suspension with antibiotic added to the suspension of bacteria and compound. Serial dilutions were performed in wells 2–10, leaving well 11 to serve as the positive control. On the same plate, a standard microdilution MIC with the tested

antibiotics and no added compound was also performed to compare the antibiotic MIC in the presence and absence of compound. The microtiter plates were covered with Glad Press-n-Seal and incubated at 37°C for 18 hr without agitation. Plates were scored by visual detection of well turbidity. Minimum inhibitory concentrations (MIC) were recorded as the lowest concentration of compound or antibiotic at which no visible bacterial growth was observed. Fold reductions were calculated by dividing the MIC of the antibiotic without compound by the MIC of the antibiotic in the presence of compound.

2.5 | Nitrocefin hydrolysis assays

For whole cell nitrocefin assays, MSSA (ATCC 29213) or MRSA (ATCC 43300) was cultured overnight in CAMHB at 37°C with shaking. The overnight culture was subcultured 1:100 in fresh CAMHB and grown at 37°C with shaking to mid-log phase ($OD_{600} = 0.4–0.6$). The culture was adjusted to an OD_{600} of 0.2 in fresh CAMHB. The suspension was aliquoted (2 ml) into sterile culture tubes and treated with compound and/or oxacillin or left untreated. These suspensions were incubated at 37°C with shaking for 30 min. The suspensions were adjusted to an OD_{600} of 0.132 in phosphate buffered saline (PBS, pH 7, 900 μl). One hundred microliters of a stock solution of nitrocefin (500 μg/ml in PBS) were added to these suspensions and mixed. The nitrocefin-bacteria suspensions (100 μl) were added to the wells of a clear 96-well polystyrene microtiter plate. A 50 μg/ml solution of nitrocefin in PBS served as a blank. Using a Biotek Synergy H1 plate reader maintained at 37°C, the absorbance at 486 nm was recorded every 5 min for 2 hr. The change in absorbance at 486 nm is expressed as a percentage of the oxacillin-induced control. The mean of three independent biological replicates, each performed with four technical replicates, is shown. Duplicate samples (before addition of nitrocefin) were serially diluted in CAMHB and plated on tryptic soy agar. The plates were incubated at 37°C overnight and the resulting colonies were enumerated and used to calculate CFU/ml. This ensured that any observed differences in β-lactamase activity were not due to the differences in CFUs.

2.6 | RNA purification

MRSA (ATCC 43300) was cultured overnight in CAMHB at 37°C with shaking. The overnight culture was subcultured 1:100 in fresh CAMHB and grown at 37°C with shaking to $OD_{600} = 0.35$. The culture was adjusted to an OD_{600} of 0.2 in fresh CAMHB. The suspension was aliquoted (2 ml) into sterile culture tubes and treated with compound and/or oxacillin or left untreated. These suspensions were incubated at 37°C with shaking for 1 hr. Triplicate cultures of each condition were briefly centrifuged and cell pellets were stored at −80°C. Pellets were resuspended in 100 μl of lyphostaphin (1 mg/ml) and incubated at room temperature for 10 min. Next, lysates were subjected to a Qiashredder column and RNA was purified with RNeasy columns according to the manufacturer's protocol (Qiagen). An on-column DNase step was also included. Total RNA was quantified and purity was assessed with a Nanodrop spectrophotometer (Thermo Scientific). RNA integrity was visualized by agarose gel electrophoresis using GelRed (Phenix) and a ChemiDoc MP (BioRad).

2.7 | RT-qPCR

200 ng of total RNA was reverse transcribed using random primers, according to the manufacturer's protocol (BioRad select cDNA synthesis kit). Minus reverse transcriptase controls were prepared for each sample. Next, duplicate qPCR reactions were performed for each cDNA template using SYBR green according to the manufacturer's protocol (BioRad SYBR Green Supermix). Primer sequences are found in Supporting Information. Those that the authors designed used NCBI Primer Blast to specify the annealing temperatures, length, location, and analyze specificity and secondary structure. All reactions were run on a StepOne thermal cycler (Applied Biosystems). Cycling parameters were as follows: 95°C for 10 min, 40 cycles of 95°C for 15 s followed by 60°C for 1 min. Specificity of each primer pair was assessed with melt curve analyses and agarose gel electrophoresis. The efficiency of each primer pair was calculated using results from calibration curves generated in Microsoft Excel. Contaminating genomic DNA levels were calculated using the comparative Ct method. Gene expression levels relative to 16S rRNA were calculated using a relative quantification model.[17] Statistical significance was determined by unpaired student's *t* tests. All calculations were performed using Microsoft Excel.

3 | RESULTS AND DISCUSSION

All compounds were evaluated at concentrations ≤25% of their MIC (Supporting Information). This ensured that any adjuvant effects were not due to combined toxic effects of the compounds with the antibiotic. Confirmation that the tested concentrations of the lead compound, amoxapine, were non-toxic was demonstrated by growth curve experiments (Supporting Information). Evaluation of structurally similar tricyclic amine antidepressants for the ability to repotentiate oxacillin revealed interesting structure–activity relationships and provided insight into the features of these molecules that are important for adjuvant activity (Table 1). Methylation of the piperazine ring caused a fourfold drop in adjuvant

TABLE 1 MIC of oxacillin in combination with tricyclic amine antidepressants in *S. aureus* 43300 (MRSA)

	+ 75 μM compound		+ 150 μM compound	
Compound	**MIC (μg/ml)**	**Fold reduction**	**MIC (μg/ml)**	**Fold reduction**
–	32	–	32	–
Amoxapine	8	4	2	16
Loxapine	32	1	8	4
Clothiapine	4	8	–[a]	–[a]
Clozapine	32	1	4	8
Olanzapine	32	1	32	1

[a]Clothiapine was not tested at 150 μM because it was not soluble at this concentration.

activity, as seen when comparing amoxapine and loxapine. However, conversion of the ether to a thioether, as with loxapine and clothiapine, provides a robust enhancement of adjuvant activity and rescues the activity lost by methylation of the piperazine ring. Clozapine displays only slightly reduced activity as compared to amoxapine, indicating that more substantive changes to the structure, including conversion of the ether to an amine and relocation of the aromatic chlorine, are well tolerated. Only olanzapine failed to exhibit any adjuvant activity at either of the tested concentrations. It is unclear from these results whether the loss of activity is due to the replacement of a benzene ring with a methylated thiophene or to the absence of an aromatic chlorine. This data provides a preliminary structure-activity relationship that highlights the importance of the bridged heteroatom in the central ring and suggests that aromatic chlorination may be important for β-lactam adjuvant activity. Methylation of the piperazine ring has a detrimental effect on the adjuvant activity, but this can be overcome with the addition of beneficial modifications.

Once we had identified that several members of the tricyclic amine class possessed adjuvant activity in MRSA, we turned our attention to understanding their mechanism of action. Using amoxapine as our lead compound, we evaluated it in combination with several cell wall active antibiotics, including β-lactams, cephalosporins, and the glycopeptide vancomycin (Table 2). Amoxapine lowered the minimum inhibitory concentration (MIC) of all tested β-lactam and cephalosporin antibiotics, but had no effect on vancomycin (Table 2). A clinically relevant community-acquired MRSA strain, USA300 as well as a methicillin-sensitive *S. aureus* (MSSA) strain, ATCC 29213, were also evaluated to assess whether amoxapine possessed broad adjuvant activity across different strains. Amoxapine reduced the MIC of oxacillin in all strains at 150 μM and at 75 μM (Table 3). This data indicated that amoxapine displayed specific potentiation of β-lactam antibiotics and that its activity was present across several strains *S. aureus*, including clinically relevant MRSA strains.

We observed that amoxapine repotentiation was limited to the β-lactam class of antibiotics as the antibiotic activity of vancomycin, also a cell wall-active antibiotic, was unchanged in the presence of amoxapine (Table 2). This led us to consider that the observed repotentiation may be due to perturbation of either β-lactamase activity or PBP2a, which are the predominant β-lactam resistance mechanisms employed in MRSA. Using a nitrocefin hydrolysis assay, we analyzed whether amoxapine affected β-lactamase activity (Figure 3a). With intact MRSA, we observed that treatment with oxacillin alone showed a marked increase in β-lactamase activity as compared to untreated cells. This was expected, due to the induction of the *bla* operon, which contains *blaZ*, the gene that encodes β-lactamase. Treatment with amoxapine alone had no effect on β-lactamase activity. When MRSA was cotreated with oxacillin and amoxapine, β-lactamase activity was dramatically reduced from levels seen with oxacillin alone. In particular, cotreatment with 150 μM amoxapine and 4 μg/ml oxacillin reduced β-lactamase activity to levels comparable to those observed in MRSA cells that have not been induced with oxacillin.

TABLE 2 MIC of antibiotics in combination with amoxapine in *S. aureus* 43300 (MRSA)

		+ Amoxapine (150 μM)	
Antibiotic	**MIC (μg/ml)**	**MIC (μg/ml)**	**Fold reduction**
Oxacillin	32	2	16
Ampicillin	32	4	8
Penicillin	16	1	16
Cefazolin	16	0.5	32
Vancomycin	2	2	1

These results suggested that amoxapine was modulating β-lactamase activity, but we questioned whether this was due to direct inhibition of β-lactamase, prevention of export from the cell, or prevention of *blaZ* gene transcription. We sought to resolve some of these questions by evaluating methicillin-sensitive *S. aureus* (MSSA) in a nitrocefin hydrolysis assay. MSSA produces endogenous β-lactamases that do not require induction with a β-lactam antibiotic and whose levels are not affected by treatment with β-lactams.

TABLE 3 MIC of oxacillin alone and in combination with amoxapine

S. aureus Strain	OxacillinMIC (μg/ml)	+ Amoxapine (150 μM) OxacillinMIC (μg/ml)	Fold reduction	+ Amoxapine (75 μM) Oxacillin MIC (μg/ml)	Fold reduction
ATCC 29213	0.25	0.125	2	0.25	1
ATCC 43300[a]	32	2	16	8	4
USA 300[a]	32	2	16	16	2

[a]Denotes methicillin-resistant strain.

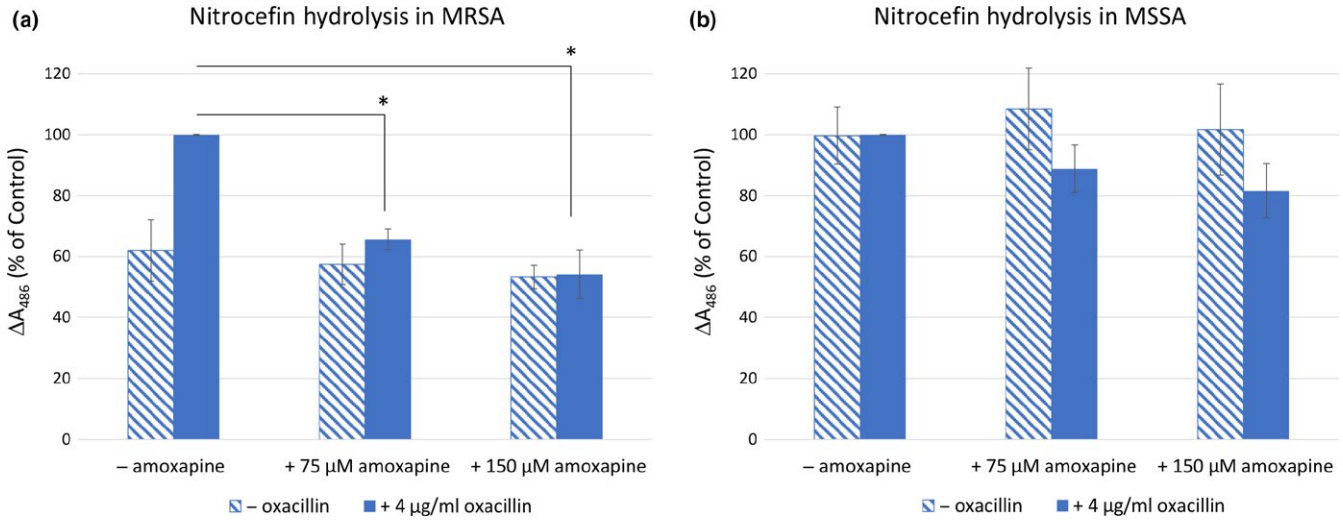

FIGURE 3 Nitrocefin hydrolysis assay. Cultures of *S. aureus* were incubated with the indicated combination of oxacillin and amoxapine for 30 min before exposure to nitrocefin, a chromogenic β-lactamase substrate. Absorbance at 486 nm was monitored for 2 hr. The change in absorbance at 486 nm is expressed as a percentage of the oxacillin-induced control. The mean of three independent biological replicates, each performed with four technical replicates, is shown. Error bars represent standard deviation. * indicates $p < 0.05$ versus the oxacillin only control. Panel (a) shows nitrocefin hydrolysis by MRSA ATCC 43300, panel (b) shows nitrocefin hydrolysis by MSSA ATCC 29213

Thus, we could analyze whether or not amoxapine was sufficient to inhibit these β-lactamases. Amoxapine alone showed no effect on β-lactamase activity as compared to untreated cells (Figure 3b). Treatment with oxacillin alone or in combination with amoxapine showed little effect on β-lactamase activity until the end of the experiment, which was likely due to cell death. Together, these data indicated that amoxapine does not directly inhibit β-lactamase activity nor does it appear to prevent β-lactamase export. This led us to conclude that amoxapine must reduce the amount of β-lactamase being produced in response to treatment with β-lactam antibiotics.

We next hypothesized that amoxapine was affecting β-lactamase production by modulating transcription of the *bla* operon and preventing the upregulation of gene transcription in the presence of β-lactam antibiotics. To test this hypothesis, we analyzed the effect of amoxapine on mRNA levels by RT-qPCR. We quantified *blaZ*, the gene that encodes for the PC1 β-lactamase enzyme, and *blaI*, the gene that encodes for BlaI, the *bla* operon transcriptional repressor (Figure 4a). As expected, treatment of cultures with oxacillin resulted in a statistically significant, 38-fold increase in *blaZ* ($p = 0.008$). Cotreatment with amoxapine and oxacillin led to a three-fold reduction in *blaZ* levels as compared to treatment with oxacillin alone ($p = 0.008$). We also observed a 13-fold increase in *blaI* levels with oxacillin treatment, but a 2.2-fold reduction in *blaI* levels with co-treatment with amoxapine and oxacillin as compared to treatment with oxacillin alone. Although it seems counterintuitive that both *blaI* and *blaZ* would be upregulated upon treatment with oxacillin, all genes in the *bla* operon are under control of the same promoter and are co-transcribed in response to β-lactam treatment.[18–20] Treatment with amoxapine alone showed no significant effect on mRNA levels as compared to the untreated control (data not shown).

As the sensory and transcription regulation of both the *bla* and *mec* operons show marked similarity, we also analyzed *mecA*, the gene that encodes modified penicillin binding

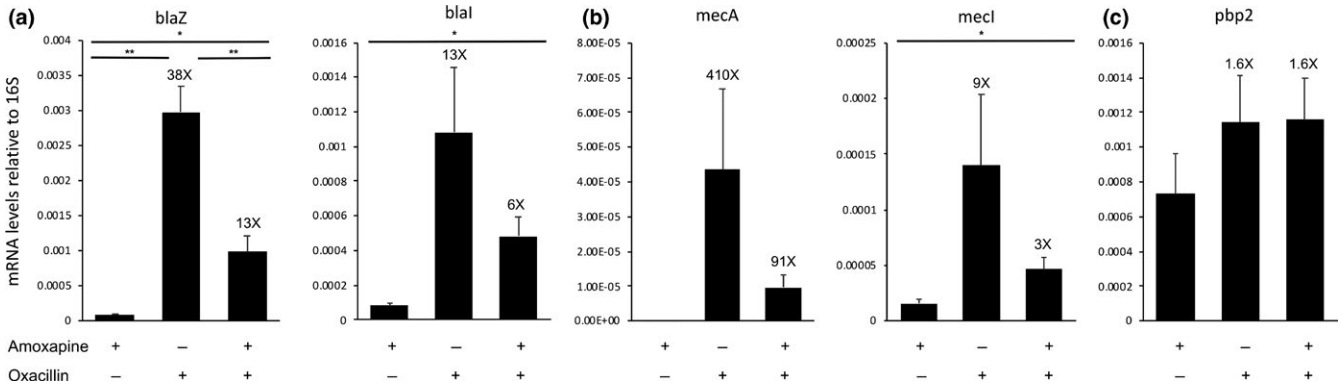

FIGURE 4 Measurement of mRNA levels in the presence and absence of amoxapine. Test genes are expressed relative to the reference gene, 16S rRNA. The mean of three independent biological replicates, each performed with two technical replicates, is shown. Error bars indicate the *SEM*. Fold changes are relative to amoxapine alone. $*p \leq 0.05$, $**p \leq 0.001$

protein PBP2a, and *mecI*, the gene that encodes for MecI, the *mec* operon transcriptional repressor (Figure 4b).[18] Interestingly, cotreatment with amoxapine and oxacillin led to a 4.5-fold reduction in *mecA* mRNA levels and a threefold reduction in *mecI* mRNA levels as compared to treatment with oxacillin alone. Cotreatment with amoxapine and oxacillin had no significant effect on the mRNA levels of *pbp2*, the gene that encodes for penicillin binding protein 2, as compared to treatment with oxacillin alone (Figure 4c). Together, these results suggest that amoxapine selectively dampens transcription of the β-lactam resistance genes *blaZ* and *mecA* in response to β-lactam exposure, thereby significantly impairing MRSA's ability to survive treatment with β-lactam antibiotics.

Although reduced mRNA levels could be attributed to increased mRNA degradation, the function of these operons points to a more likely decrease in transcription of the *bla* and *mec* operons. Additionally, other small molecule adjuvants with similar mechanisms of action have been described. FDA-approved phenothiazines, including thioridazine and chlorpromazine, similarly repotentiate MRSA to β-lactam antibiotics by inhibiting *blaZ* and *mecA* gene transcription in the presence of β-lactam antibiotics.[21]

4 | CONCLUSIONS

In summary, we have identified structural features that both enhance and impair the β-lactam adjuvant activity of the investigated tricyclic amine antidepressants. This provides a focused structure–activity relationship that can guide synthetic efforts toward more potent adjuvants. Furthermore, we have shown that the lead compound, amoxapine, simultaneously and selectively reduces the mRNA levels of the *blaZ* and *mecA* operons upon cotreatment with oxacillin.

Small molecules, such as amoxapine, that concurrently inhibit both major β-lactam resistance mechanisms in MRSA, have obvious utility as antibiotic adjuvants. Unfortunately, the known psychoactivity of these tricyclic amine compounds and the effective concentrations prevent their immediate and direct use in the clinic. However, this compound class is ripe for further development and study. Synthetic efforts to generate more potent analogs for further study as well as biochemical studies to identify the molecular target(s) of these molecules are underway in our laboratory and will be reported in due course.

ACKNOWLEDGMENTS

This work was fully funded by High Point University (High Point, NC). The authors would particularly like to thank Research and Sponsored Programs (MSB), the Summer Undergraduate Research Program in the Sciences (KRG, HBM, MSB), and Undergraduate Research and Creative Works (KRG, HBM, MSB) for their generous support.

CONFLICT OF INTEREST

The authors declare the following competing financial interest: MSB and HBM have filed a patent on the technology disclosed.

ORCID

Heather B. Miller http://orcid.org/0000-0003-1400-7716
Meghan S. Blackledge http://orcid.org/0000-0001-9632-0567

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

How to cite this article: Gillard K, Miller HB, Blackledge MS. Tricyclic amine antidepressants suppress β-lactam resistance in methicillin-resistant *Staphylococcus aureus* (MRSA) by repressing mRNA levels of key resistance genes. *Chem Biol Drug Des*. 2018;00:1–8. https://doi.org/10.1111/cbdd.13361