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Resensitization of methicillin-resistant *Staphylococcus aureus* by amoxapine, an FDA-approved antidepressant

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Abstract

The rapid increase in bacterial resistance to antibiotics is a global healthcare crisis. Non-antibiotic pharmaceuticals that have attained approval by the United States Food and Drug Administration have the potential to be repurposed as bacterial resistance-modifying agents and therefore could become valuable resources in our battle against antibiotic-resistant microbes. Amoxapine is a tetracyclic antidepressant used in the treatment of major depressive disorder. Here we demonstrate the ability of amoxapine to resensitize methicillin-resistant *Staphylococcus aureus* strain ATCC 43300 to oxacillin in both agar diffusion and broth microdilution assays. Amoxapine also reduced the bacterial cleavage of nitrocefin in a dose-dependent manner, suggesting that it may exert its adjuvant effects through reduction of beta-lactamase activity.

Keyword: Microbiology

1. Introduction

Methicillin-resistant *Staphylococcus aureus* (MRSA) is responsible for an increasingly large number of infection-related illnesses and deaths each year. In 2015, MRSA was responsible for over 11,000 deaths in the United States alone [1].

The development of antibiotic resistance by bacteria is an inevitable consequence of antibiotic use and the process of natural selection, however the spread of resistance is likely accelerated by the frequent overuse and misuse of medically valuable antibiotic compounds [2, 3]. While the prevalence of antibiotic resistance in *S. aureus* and other pathogenic bacteria is growing, efforts to discover and develop novel antibiotic compounds remains a lengthy, laborious, and costly endeavor [4]. This combination of scientific, economic, and regulatory challenges has resulted in a dramatic reduction in the number of antibiotic compounds approved by the United States Food and Drug Administration (FDA) over the last 3 decades, causing the World Health Organization and others to warn of an impending post-antibiotic era [5, 6, 7].

Rather than developing novel antibiotic compounds that will inevitably be rendered ineffective by the rise of bacterial resistance, some researchers and pharmaceutical companies have explored alternative strategies to combat antibiotic-resistant microbes. One strategy is to develop formulations that include adjuvant compounds, which increase the efficacy of existing antibiotics [8, 9, 10, 11]. Each of the thousands of FDA-approved pharmaceutical compounds has the potential to act as a resistance-modifying agent, and yet most remain underutilized resources for adjuvant molecule exploration. The existing compounds of the FDA-approved pharmacopeia also have well-established toxicity profiles and are relatively inexpensive compared to *de novo* drug development. The exploration of existing drugs for repurposing as antibiotic adjuvants has the potential to provide an additional line of defense in the antibiotic resistance crisis and ultimately could save countless lives [12, 13].

Amoxapine (Fig. 1A) is a tricyclic antidepressant of the dibenzoxazepine class that was developed in the early 1970s and marketed under the brand names of Asendin, Asendis, Defanyl, and Demolox [14]. It continues to be prescribed to treat the symptoms of depression. Here we demonstrate the potential for amoxapine to act as a resistance-modifying agent for MRSA strain ATCC 43300. At sublethal concentrations, amoxapine potentiates the activity of oxacillin against *S. aureus* in both agar diffusion and broth dilution assays. We also demonstrate that amoxapine reduces the cleavage of nitrocefin, a chromogenic beta-lactam compound, by MRSA strain ATCC 43300.

2. Materials and methods

2.1. Bacterial strains and media

Bacterial strains used in the study were methicillin-sensitive *S. aureus* (MSSA) strain ATCC 29213 and MRSA strain ATCC 43300 (ATCC, Manassas, VA, USA). Bacteria were cultured on Muller Hinton II broth (MHB) or agar (MHA) at 37 °C (Becton Dickson and Company, Sparks, MD, USA).
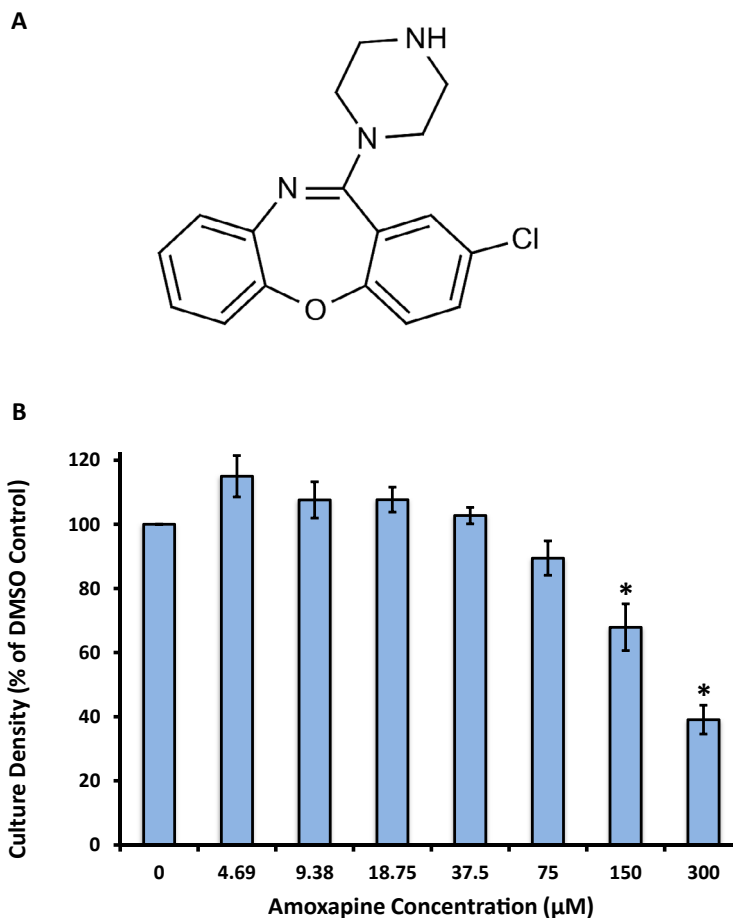
A

B

Fig. 1. Growth of *S. aureus* is not completely inhibited by amoxapine. (A) Chemical structure of amoxapine, CAS 14028-44-5. (B) Optical densities (OD_{600}) of 3 mL *S. aureus* cultures after 16–18 h of incubation at 37 °C with shaking at 180 rpm in the presence of amoxapine. Culture densities are expressed as a percentage of the DMSO control. The mean of 4 technical replicates collected from 2 independent experiments is shown. Error bars represent SEM. * indicates $p < 0.05$ vs DMSO control.

2.2. The effect of amoxapine on the growth of *S. aureus*

Amoxapine, CAS number 14028-44-5 (TCI, Tokyo, Japan) stocks were prepared at 30 mM in DMSO. In an effort to limit the effect of DMSO on bacterial growth, the final solvent concentration was maintained at 1% (v/v) for all experimental and control cultures. An overnight culture of MSSA was diluted to 2×10^6 CFU/mL in 3 mL cultures of MHB containing 1:2 serially diluted concentrations of amoxapine ranging from 300 μM to 0.59 μM or 1% DMSO without amoxapine. Each experimental condition was prepared in duplicate. Following 16–18 h of incubation at 37 °C with continuous shaking at 180 RPM, the turbidity of each culture was assessed by measuring OD_{600} and expressed as a percentage of the solvent control.

2.3. Agar diffusion assay with amoxapine-infused plates

Following autoclave sterilization, flasks of MHA were allowed to cool to approximately 60 °C. Either DMSO or amoxapine was aseptically added to the liquid, mixed on a magnetic stir plate to ensure even distribution, and poured into 100 mm plastic petri dishes. Control plates contained 1% (v/v) DMSO and amoxapine-infused plates contained 300 µM amoxapine and 1% (v/v) DMSO. Overnight cultures of MSSA and MRSA were diluted to approximately 5×10^8 CFU/mL in fresh MHB and plated as a continuous lawn using sterile polyester-tipped applicators. Following inoculation, oxacillin-impregnated (1 µg, OX 1) Sensi-Disc (Becton Dickson and Company, Franklin Lakes, NJ, USA) were applied to the agar surface with gentle pressure. Following 16–18 h of incubation at 37 °C, the diameter of the zones of inhibited bacterial growth were measured using digital calipers.

2.4. Broth microdilution assay

Oxacillin (Acros Organics, Thermo Fisher Scientific, Waltham, MA, USA) minimum inhibitory concentrations (MIC) were determined in the presence and absence of amoxapine using the broth microdilution assay described by the Clinical and Laboratory Standards Institute [15]. Briefly, a 1:2 dilution series of oxacillin was prepared in 4 concentrations of amoxapine. Final concentrations of amoxapine were 0 µM, 75 µM, 150 µM, and 300 µM in 1% DMSO (v/v). Lastly, overnight cultures of MSSA and MRSA were diluted in MHB and inoculated to a final concentration of 5×10^4 CFU/well. The total volume in each microtiter well was 100 µL. MICs were determined by visual inspection following 16–18 h of static incubation at 37 °C. Using unaided eye observation, we determined the MIC to be the lowest concentration of oxacillin that completely inhibited bacterial growth [15]. Each experimental condition was prepared in duplicate.

2.5. Nitrocefin cleavage assay

In order to increase the bacterial production of beta-lactamase prior to our assay, a culture of MRSA, freshly diluted to 2×10^8 CFU/mL, was incubated for 30 minutes at 37 °C with shaking at 180 RPM in the presence of 10 µg/mL oxacillin. The cells were washed 3 times by low speed centrifugation and resuspension in 0.1 M potassium phosphate buffer, pH 7.0 to remove the oxacillin. The concentration of washed cell suspensions was adjusted to provide 1×10^7 CFU/well. The final reaction volume of 100 µL contained 1×10^7 CFU/well MRSA, 50 µg/mL nitrocefin (Oxoid, Thermo Fisher Scientific, Waltham, MA, USA), 0 µM, 75 µM, 150 µM, or 300 µM amoxapine, and 1% DMSO (v/v). Five technical replications of each experimental condition were included on each microtiter plate. Cleavage of nitrocefin was monitored in a Synergy H1 microplate reader (BioTek Instruments,

Winooski, VT, USA) by measuring OD_{486} for 2 h at 37 °C with 5 s of shaking prior to each absorbance reading. Starting absorbance values were subtracted from the 2 h absorbance values to derive ΔOD_{486}.

2.6. Statistics

Two-tailed Student's t-test analyses between control and treatments were performed using Microsoft Excel. We judged statistical significance to be $p <$ 0.05. Error bars represent standard error of the mean.

3. Results

3.1. Amoxapine treatment does not completely inhibit the growth of *S. aureus*

In order to determine the effect of amoxapine treatment on the growth of MSSA in broth culture, we conducted a series of 1:2 serial dilutions of amoxapine in a final concentration of 1% DMSO. Following 16–18 h of growth, both the 150 μM and 300 μM conditions exhibited statistically significant reductions in final culture density (OD_{600}) compared to the DMSO control (Fig. 1B). Importantly, while 300 μM amoxapine did cause a 60% reduction in the growth of *S. aureus* in broth culture, it did not completely inhibit bacterial growth, and thus, we did not determine the minimum inhibitory concentration for amoxapine in this study.

3.2. Amoxapine increases the sensitivity of *S. aureus* to oxacillin in an agar diffusion assay

We used a modified disc-diffusion assay to explore the potential of amoxapine to sensitize *S. aureus* to the beta-lactam antibiotic oxacillin. As our amoxapine titration experiment failed to establish a concentration that completely inhibited bacterial growth, we incorporated amoxapine into MHA plates at a final concentration of 300 μM, the highest concentration tested in our study. For both MSSA and MRSA, the oxacillin zone of inhibition was significantly larger on the amoxapine-containing plates compared to the DMSO-containing control plates (Fig. 2A). Relative to MSSA, the effect of amoxapine was more robust in MRSA, more than doubling the average zone of inhibition from 9.4 mm to 18.9 mm.

3.3. Amoxapine increases the sensitivity of MRSA to oxacillin in a microdilution assay

To better quantify the impact of amoxapine on the sensitivity of MSSA and MRSA to oxacillin, we conducted static microdilution assays in 96-well microtiter plates. We determined the MIC of oxacillin in 3 concentrations of amoxapine (Table 1). In the absence of amoxapine, MRSA is highly resistant to oxacillin, with an MIC of
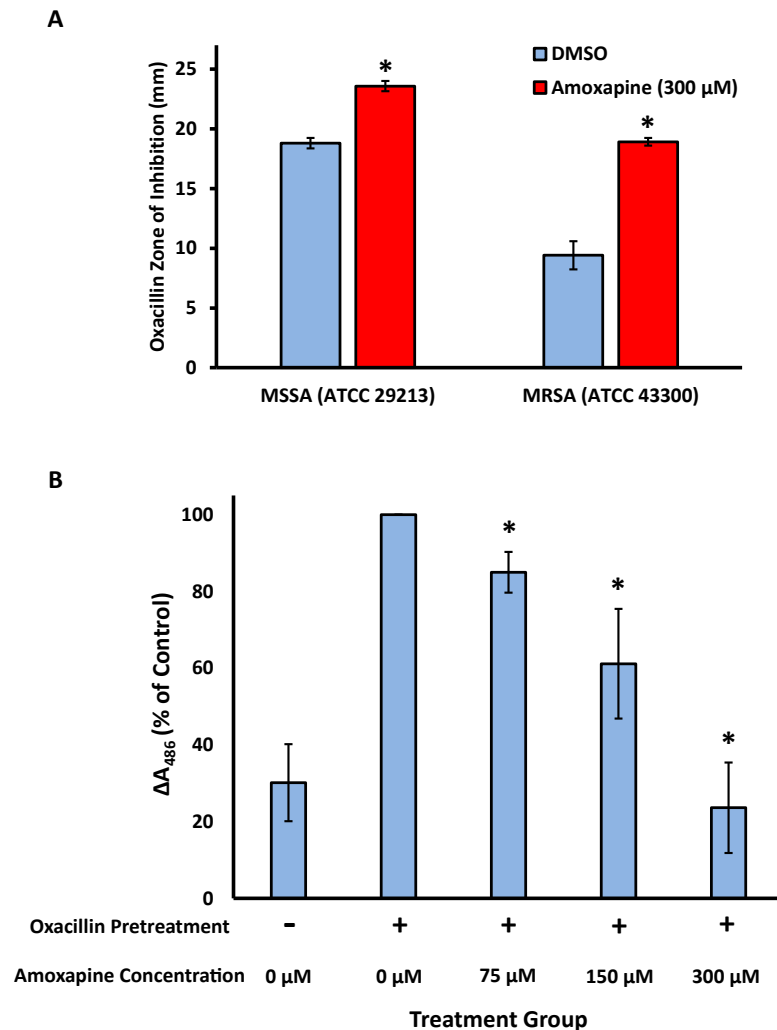
Fig. 2. Effects of amoxapine on oxacillin disc-diffusion sensitivity and nitrocefin cleavage. (A) Zones of inhibited methicillin-sensitive *S. aureus* (MSSA) and methicillin-resistant *S. aureus* (MRSA) growth by oxacillin. MHII agar plates contained either 1% DMSO (blue bars) or 300 μM amoxapine (red bars). For MSSA, the mean of 3 technical replicates collected from a single experiments is shown. For MRSA, the mean of 5 technical replicates collected from 2 independent experiments is shown. (B) Following a pretreatment with oxacillin to increase β-lactamase production, washed cultures of MRSA were incubated at 37 °C in the presence of amoxapine and nitrocefin, a chromogenic β-lactamase substrate in a 96-well microtiter plate. Absorbance of the cultures at 486 nm was monitored for a period of 2 h. The change in absorbance at 486 nm is expressed as a percentage of the oxacillin-induced DMSO control. The mean of 4 independent biological replicates, each performed with 5 technical replicates, is shown. Error bars represent SEM. * indicates $p < 0.05$ vs DMSO control.

16 μg/mL. Increasing amounts of amoxapine resulted in a dose-dependent resensitization of MRSA to oxacillin. The presence of 150 μM amoxapine reduced the oxacillin MIC 8–16 fold from the DMSO control to 1–2 μg/mL, and 300 μM amoxapine reduced the oxacillin MIC 128 fold to 0.125 μg/mL. In contrast to the MRSA, treatment of MSSA did not result in a robust reduction of oxacillin MIC

Table 1. Minimum inhibitory concentrations of oxacillin against methicillin-sensitive and methicillin-resistant *S. aureus* in the presence of amoxapine. Data represent the results of 3 independent experiments performed in duplicate. An MIC range is presented for conditions in which results varied.

Amoxapine Concentration	MSSA (ATCC 29213) Oxacillin MIC (µg/ml)	MRSA (ATCC 43300) Oxacillin MIC (µg/ml)
0 µM	0.25	16
75 µM	0.25	4–8
150 µM	0.25	1–2
300 µM	0.125	0.125

Data represent the results of 3 independent experiments performed in duplicate. An MIC range is presented for conditions in which results varied.

values (Table 1). The presence of 300 µM amoxapine reduced the oxacillin MIC of MSSA 2 fold from the DMSO control, but the 150 µM and 75 µM amoxapine treatments had no effect on oxacillin MIC.

3.4. Amoxapine reduces the cleavage of nitrocefin by MRSA

One common mechanism of microbial resistance to beta-lactam antibiotics is mediated through bacterial synthesis and secretion of beta-lactamase enzymes into the extracellular environment. Thus, we sought to investigate the potential contribution of beta-lactamase inhibition to our observed amoxapine-mediated increase in MRSA oxacillin sensitivity. We measured extracellular beta-lactamase activity by monitoring the cleavage of nitrocefin, a chromogenic cephalosporin that serves as an enzymatic substrate. Nitrocefin contains a beta-lactam ring that is susceptible to hydrolysis by beta-lactamase enzymes. This cleavage causes a shift in the light absorbance properties of nitrocefin, allowing the detection of beta-lactamase activity by monitoring the change in absorbance at 486 nm.

Prior to amoxapine exposure, we induced elevated expression of beta-lactamase enzymes by treating MRSA cultures with oxacillin for 30 min. Pretreatment with oxacillin approximately tripled the amount of extracellular beta-lactamase activity (Fig. 2B, column 1 and 2). Following the removal of oxacillin by 3 rounds of centrifugation and resuspension in phosphate buffer, amoxapine and nitrocefin were added to the cultures simultaneously. The presence of amoxapine caused a statistically significant, dose-dependent reduction in the cleavage of nitrocefin compared to the DMSO control over 2 h of incubation (Fig. 2B).

4. Discussion

Amoxapine causes a reduction in the growth of *S. aureus* at concentrations greater than or equal to 150 µM (Fig. 1B), however it does not completely inhibit growth

at 300 μM, the highest concentration tested our studies (Figs. 1 and 2 and Table 1). While amoxapine did not completely inhibit bacterial growth, the antidepressant compound did exhibit the ability to suppress oxacillin resistance in both agar diffusion and broth microdilution assays (Table 1, Fig. 2A). The ability of 300 μM amoxapine to reduce MRSA sensitivity to oxacillin by 128 fold to 0.125 μg/mL is particularly noteworthy, as the amoxapine-sensitized MIC falls well below the 2 μg/mL oxacillin MIC breakpoint for MSSA [16].

The mid-micromolar concentrations of amoxapine (75–300 μM) required to confer adjuvant activity in our studies are much higher than the peak serum/plasma concentrations in human subjects, which are in the nanomolar range [17, 18, 19]. Additionally, these concentrations of amoxapine are elevated compared to the effective concentrations of previously described compounds that increase the efficacy of β-lactam antibiotics *in vitro*; approximately 10–20 μM of clavulanic acid, sulbactam, or tazobactam is sufficient to potentiate the action of amoxicillin against *S. aureus* [20].

Our use of mid-micromolar concentrations of amoxapine increase the possibility of non-specific bacterial sensitization. However, two lines of evidence suggest that amoxapine's resensitization of MRSA functions through a resistance-related mechanism. Firstly, the adjuvant effect of amoxapine on MRSA was far more robust than its effects on MSSA in both the agar diffusion and microdilution assays (Table 1, Fig. 2A). Secondly, the nitrocefin cleavage assay demonstrates that amoxapine is exerting resistance modification activity, in part, by interfering with some aspect of the bacterial beta-lactamase system. Amoxapine clearly reduced the cleavage of nitrocefin, a cephalosporin compound containing a beta-lactam ring, in a dose-dependent manner (Fig. 2B) and thus, may be acting as a direct inhibitor of beta-lactamase, as is the case with previously described adjuvant compounds: clavulanic acid, sulbactam, and tazobactam [8]. However, due to our measurement of nitrocefin cleavage by intact MRSA rather than cell lysate or purified beta-lactamase enzyme, we did not determine the specific mechanism by which amoxapine inhibits nitrocefin cleavage. Therefore, we cannot rule out inhibition of beta-lactamase synthesis or export as potential amoxapine modes of action.

Finally, this study included a single strain of MRSA (ATCC 43300) and therefore may not be applicable to all MRSA strains. The future exploration of additional MRSA strains with diverse mechanisms of resistance would provide valuable insights into the mechanism of amoxapine-mediated resensitization.

5. Conclusion

Despite an undefined mode of action, amoxapine exhibits promise as a resistance-modifying agent for MRSA and warrants further exploration in other strains of

antibiotic-resistant bacteria. Further exploration of tetracyclic antidepressants could also yield exciting molecules with adjuvant activity.

Declarations

Author contribution statement

Tyler J. Wilson: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data; Wrote the paper.

Meghan S. Blackledge: Conceived and designed the experiments; Contributed reagents, materials, analysis tools or data; Wrote the paper.

Patrick A. Vigueira: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data; Wrote the paper.

Competing interest statement

The authors declare no conflict of interest.

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Additional information

No additional information is available for this paper.

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