Pyrimidine-2-amines increase susceptibility of methicillin-resistant *Staphylococcus aureus* to penicillin G

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ABSTRACT

β-lactamase (penicillinase) based resistance renders early β-lactams like penicillin G useless against methicillin-resistant *Staphylococcus aureus* (MRSA). Finding novel antimicrobials is difficult, and resistance has been observed against most treatment options. Increasing the susceptibility of MRSA to early β-lactams would help bring back the clinical utility of a powerful and safe class of antimicrobials. Moreillon and coworkers have already demonstrated that β-lactamase inhibition can render penicillin G more powerful than oxacillin. Yet, they also highlighted that direct β-lactamase inhibitors are not the best tool for the job. Here, we reveal that certain pyrimidin-2-amines (P2A) reduce the minimum inhibitory concentration (MIC) of penicillin G against resistant *S. aureus* strains reliably by up to 64-fold, when present at 50 μM. P2As do not inhibit β-lactamases directly, but reduce its expression; This is in stark contrast to direct penicillinase inhibitors like sulbactam and clavulanate that covalently bind the secreted protein. The MIC of penicillinase-insensitive oxacillin were not altered, clearly because these cannot overcome PBP2a; This demonstrates the advantage of penicillin G over penicillinase-insensitive β-lactams. Gene knockout experiments show that factors commonly associated with decreased resistance to cell wall-active antimicrobials, like Stk1 and VraS, are not the target of P2As. Yet, multiple gene knockouts were resistant to P2A activity, suggesting these chemicals act through an as-yet-unknown central controller. A preliminary structure-activity relationship has also provided insights into pharmacophoric features. We have demonstrated that Moreillon's principle, that penicillin G is useful against MRSA upon cessation of β-lactamase activity, can be practically implemented by suppression of penicillinase expression instead of direct β-lactamase inhibition.

INTRODUCTION

Antimicrobial resistance (AMR) has emerged in *Staphylococcus aureus* stepwise (reviewed[1]). The introduction of penicillins such as penicillin G in the 1940s led to penicillinase-based resistance. Upon the introduction of penicillins that are resistant to penicillinases – such as methicillin and oxacillin – methicillin-susceptible strains (MSSA) gave way to MRSA. MRSA express both, PBP2a as well as penicillinases.[1, 2] MRSA are, consequently, resistant to most β-lactams. Vancomycin – once considered a "drug of last resort" – became the frontline therapy against MRSA,[3] giving rise to vancomycin-intermediate resistant (VISA) strains.[4] VISA isolates are often also resistant to β-lactams,[5] making antimicrobials such as oxazolidinones (e.g., linezolid), and streptogramins (e.g., quinupristin-dalfopristin) frontline treatments. As can be expected, resistant cases[6-9] are emerging against these and other treatment options. Resistance will only increase with antimicrobial use.[10]

Community- and hospital-acquired *S. aureus* infections are common today.[11] Community-acquired MRSA infections are widespread now due to a variety of reasons, including the current opioid crisis and the shared use of dirty needles.[12, 13] For instance, in North Carolina alone, the number of infective endocarditis cases increased 20-fold across a 5-year span, with a concomitant increase in financial burden for society. Since *S. aureus* is a major etiological agent in infective endocarditis,[3] a large part of this burden can be attributed directly to antimicrobial resistance in this pathogen. We need novel ways of fighting *S. aureus* infections.

While antimicrobial discovery is still the focus of our attempts at solving the resistance problem, non-traditional options are also being considered. Identifying enhancers[14] (chemicals that increase the potency of clinically relevant antimicrobials) has already resulted in approved, marketed drugs such as Augmentin™,[15] a combination of amoxicillin with clavulanate; Clavulanate inhibits penicillinases, preventing the deactivation of amoxicillin, thus making it more effective against β-lactamase producing MSSA. These successes at devising enhancers are highly promising, even if Augmentin is not clinically useful against MRSA infections due to the expression of PBP2a, whose expression is widely expected[16] to overcome any β-lactam except ceftaroline. On the other hand, Moreillon and others have put forward strong evidence[17, 18] that older, natural penicillins and aminopenicillins have ~10-fold more affinity for PBP2a than semi-synthetic penicillins such as oxacillin, if penicillinase is not present. They also found that these penicillins sterilize infective endocarditis vegetations in rabbits within 4 days of therapy if penicillinase was ineffective. Penicillin G alone was as effective against penicillinase knockout MRSA infections as vancomycin was against wild-type infections.[19] Penicillin G was actually more effective than vancomycin against the penicillinase negative MRSA strain.[19] This is critical because vancomycin is first-line therapy for MRSA infections. However, treatment of infected animals with a combination of high-dose sulbactam with penicillin G was associated with failures due to resistant strains. It is evident that covalent mechanism of penicillinase-based penicillin inactivation will reduce the amount of the antimicrobial available to act on its target, when agents like sulbactam or clavulanate are used.[18] As a result, resistant mutants are able to survive. Notably, *blaZ* knockout strains

did not demonstrate similar failures or resistance. In light of the above, our hypothesis is that inhibiting β-lactamase expression will enhance the potency of penicillinase-sensitive penicillins against MRSA. This suggests that *blaZ* suppression is an alternative to direct penicillinase inhibitors like sulbactam and clavulanate. If successful, this adaptation of Moreillon's strategy[20] could help revive the clinical utility of natural penicillins, which are currently considered of no clinical utility against this pathogen.

Here, we report the discovery of P2As as a novel class of chemical enhancers that re-sensitize MRSA to penicillin G by up to 64-fold by suppression of β-lactamase gene expression. The structure of the prototype P2A is shown in **Fig 1**. These chemicals are relatively small (~350-400 Da) and hence have high potential for further optimization and development. Limited structural changes could be made to the core motif, suggesting P2As function at a specific binding pocket. Little to no activity was observed in altering the MIC of penicillin G against VISA, so the phenotype is specific to MRSA as well. All this suggests specific modulation of a single target, although further investigation is needed to establish this beyond doubt.

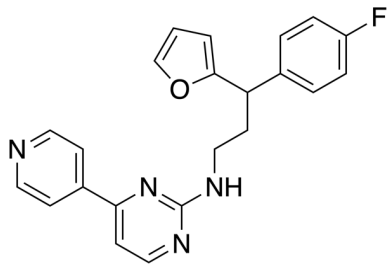


Figure 1. *The structure of the prototype P2A (**1**) that enhances the potency of penicillin G against USA300 MRSA strain ATCC BAA-1717. The chemical was in its racemic form, as were all chiral center-containing structural analogs tested during this study.*

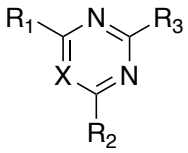
Our chemicals re-sensitize MRSA to penicillin G, but not penicillinase-resistant antimicrobials such as oxacillin. Recent literature reports[21-24] on enhancer discovery have focused on 2 central pathways controlling oxacillin resistance: A eukaryotic-like serine/threonine kinase called Stk1[25-27] and a histidine kinase called VraS.[28-39] We will show that P2As do not function via these pathways, suggesting their target is novel. Our finding that P2As reduce the MIC of penicillin G, but not oxacillin and other penicillinase-insensitive β-lactam antimicrobials, demonstrates the feasibility of bringing the oldest of our arsenal of antimicrobials back into use.

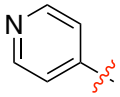
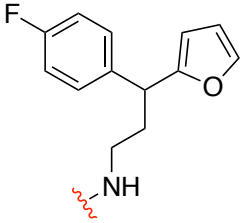
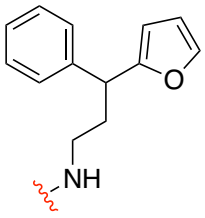
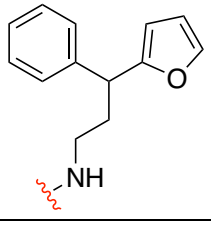
RESULTS AND DISCUSSION

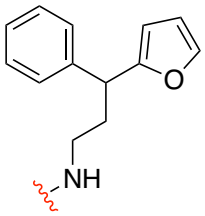
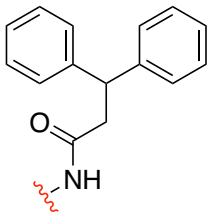
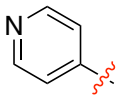
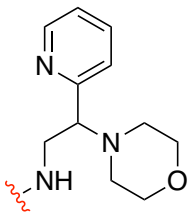
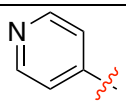
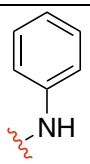
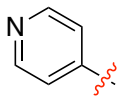
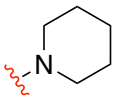
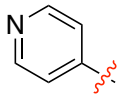
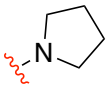
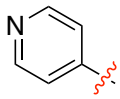
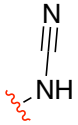
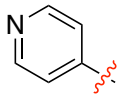
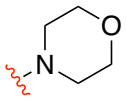
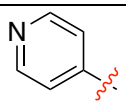
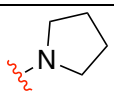
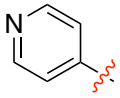
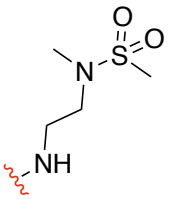
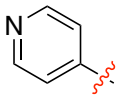
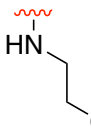
P2As are potent enhancers of penicillin G against MRSA. Certain P2As are able to reduce the MIC of penicillin G against MRSA strain ATCC BAA-1717 (**Table 1** and **Supplementary Table S1**). The MIC of penicillin G was ~256 μg/mL, but addition of 50 μM chemical **1** enhanced the potency by at least 8-fold. A titration showed that at least

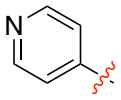
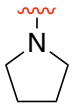
50 μM P2As was necessary to induce ≥4-fold MIC reduction for penicillin G, so these are only early hits, and will require significant optimization. P2As are able to reduce the MIC of vancomycin against VISA strain Mu50, as well. Vancomycin showed an MIC of ≤2 μg/mL against VISA in the presence of 50 μM chemical **1**, when its MIC was 8 μg/mL when treated with DMSO control (**Supplementary Table S2**). While this is a promising find, we will focus on MRSA and penicillin G in this manuscript.

Table 1. *Changes in chemical structure demonstrates varied ability to enhance penicillin G potency against USA300 MRSA ATCC BAA-1717. Biological replicates were run only for the promising chemicals (**1**, **2** and **3**). The full range is reported if different between the two replicates.*



Chemical	R_1	R_2	R_3	X, if not (CH)	Penicillin G potency in presence of 50 μM P2As	
					MIC (μg/mL)	Fold drop in MIC
DMSO control					256	-
1					32	8
2					4, 32	8 to 64
3					8, 32	8 to 32

4				N	128	2
5					256	
6					128	2
7					128	2
8					>256	
9					128	2
10					128	2
11					128	2
12					128	2
13					>256	
14					128	2

15					256	

Recent studies have reported enhancement of oxacillin potency against MRSA strains by inhibition of Stk1 and VraS.[21-24] Our findings were different, in comparison. P2As are unable to reduce the MIC of oxacillin against MRSA strain ATCC BAA-1717 (**Table 2**). The presence of P2As did not alter the MIC of other antimicrobials, such as oxacillin, chloramphenicol and vancomycin against MRSA.

Table 2. Effect of the prototype P2A (**1**) on penicillinase-resistant β-lactams and other antimicrobials. Further details can be found in **Supplementary Table S3**.

	MIC (μg/mL)	
antibiotic	**+ DMSO**	**+ 50 μM P2A *1***
oxacillin	128	256
chloramphenicol	32	32
vancomycin	1	1

Based on the above, we hypothesized that P2As function by either inhibiting penicillinases or else their expression by MRSA. **Table 3** demonstrates that penicillinase activity of live MRSA in a nitrocefin assay was indeed reduced. At the same time, a control experiment showed that **1** did not inhibit purified β-lactamase (**Supplementary Table S4** and **Supplementary Fig S1**). The rate of nitrocefin hydrolysis was indistinguishable between two samples treated with either **1** or control. This conclusively demonstrates P2As function by suppressing penicillinase production, and not by direct neutralization.

Table 3. Nitrocefin assay demonstrates P2As reduce penicillinase activity of MRSA strain ATCC BAA-1717. MRSA was incubated with chemicals or DMSO control and centrifuged. Supernatants were treated with nitrocefin and color development was monitored at 486 nm. See Materials and Methods and **Supplementary Table S5** for details.

Chemical @ 50 μM	**% β-lactamase activity when compared with DMSO control**
Loratadine	9.6±0.04
1	23.95 ± 15
2	48.8 ± 2.1
3	78.75 ± 5.25

P2As bind a hypothetical, tight pocket. We have performed structure activity work (**Table 1** and **Fig 2**) to explore the space around the P2A scaffold. The pyridinyl- group at the R1 position of **1** is well tolerated, but the methylamino- substituent at the same position retains potency in **2**. **3** has a methoxy- substitution, and is as potent as **1** and **2**. **2** and **3** may even be marginally superior to **1**, resulting in a potency increase of up to 4- to 8-fold in comparison, although there was clearly some variability in our results. **4** differs from **1**-**2** in having no substitution at R1, but loses the ability to enhance penicillin G

activity. **5**, on the other hand, has a methyl- substituent at R1, and also fails to enhance penicillin G potency. Comparing **1** to **5**, it seems there is an electrophilic binding partner present near the R1 position, with a propensity to bond with a nitrogen than oxygen. **4** also possesses an additional nitrogen in the core ring, making it a triazine instead of a diazine, but this does not affect activity.

Chemicals **6**-**9** comprise the same substituents as **1** at R1 and R2, but differ at R3. None of **6**-**9** are able to enhance the MIC of penicillin G as well as **1**; So, they help us understand the nature of the binding pocket at the R3 position. The R3 substituents are all hydrophobic, nitrogen-containing heterocyclic substituents. The 2-morpholino-2-(pyridine-2-yl)-ethyl-1-amino- substituent on **6** represents the structure closest to **1**, even though effectually, it is shorter by one carbon and contains an aliphatic morpholine instead of a furan. **7**, **8** and **9** have phenylamino-, piperidinyl-, and pyrrolinyl- substituents at R2, representing a shrinking substituent. Thus, comparing **6**-**9** with **1** suggests R3 binds to a tight pocket, almost like a narrow tunnel ending in a wide cavity. **10** is different from **6**-**9** because of a cyanamide substituent at R3, which is rigid due to the sp3 carbon and nitrogen, but still lacks penicillin-enhancement activity, perhaps because it would not fit into this tunnel due to its rigid, linear nature.

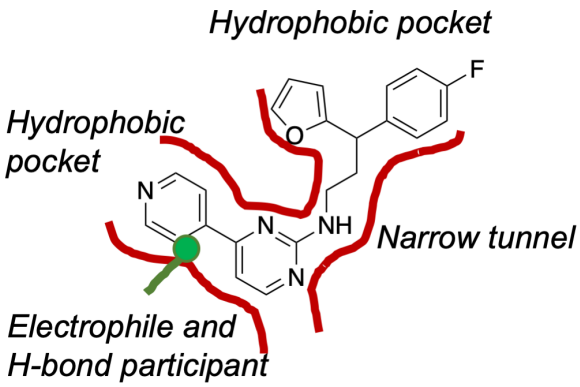


Figure 2. A hypothetical binding pocket for P2As. *The P2A core binds in the main pocket, while the R1 substituent binds in a narrow groove possessing an electrophile. The nature of the R2 group-binding region is uncertain, but small groups have been tolerated well. In comparison, our observations suggest the R3 groups bind in an elongated, narrow tunnel that opens up to a wider groove.*

A few of our chemicals have concurrent substitutions at multiple positions in comparison with **1**, which could explain the change in activity. **11** possesses a morpholino- group at R3 and an ethyl group at R2, even if it is indistinct from **1** otherwise. Its inability to enhance penicillin G activity could be due to both, R2 and R3 substituents. Likewise, the methyl- at R2 or the pyrrolinyl-group at R3 could contribute to reduced activity of **12**. **13** represents a flexible 2-(N-methyl, N-methylsulfonyl)amino-ethyl-1-amino- R3 substituent. **5** has a methyl group at R1, and a 3,3-diphenyl-propionylamide group at R3; It also fails to enhance the MIC of penicillin G, although it is uncertain whether this is due to differences at the R3 or R1 position. Most likely, a combination of both factors. **13**, combined with **5**, seem to indicate that simply having a flexible, hydrophobic substituent at R3 is inadequate – the 3-(furan-2-yl)-3-phenylpropyl-1-amino-

substituent present in **1**-**4**, present at R3, seems to be required from our small sample of chemicals tested. **14** and **15** explore the R2 position with a flexible 2-hydroxy-ethyl-1-amino- and a cyclic 1-pyrrolinyl- substituents that did not increase activity; both possess an unionizable amino group at R3. Neither is able to enhance the MIC of penicillin G. This could simply be due to lack of binding or failure to enhance binding – we cannot tell at this time. It should also be mentioned that, at least in theory, the R2 and R3 substituents in **14** and **15** could flip, which would explain the lack of activity of **15** (failure of a short, bulky group to bind the narrow R2 tunnel).

As is evident, we have not yet explored the P2A moiety itself. Commercially available chemicals have allowed us to explore the hypothetical P3 pocket reasonably, but we will need significant synthetic effort to modify our narrow range of active chemicals (**1**, **2** and **4**), which remains a future goal. Ultimately, this is only a preliminary report on developing some structure-activity data, and a more detailed exploration will be necessary to truly develop these observations into something conclusive, and to eventually help identify leads.

Overall, the current data strongly suggests 1-3 are the representative P2As for hit-to-lead optimization, as most other structural alterations reported in this manuscript led to a complete loss of activity. Therefore, we have reported further characterization only for these chemicals.

Knockout studies demonstrate that Stk1 and VraS are not the targets of P2As. We tested the NTML library of MRSA mutants to identify potential pathways involved in P2A activity. In particular, we are interested in kinases like Stk1[21, 22, 25, 27, 40] and VraS, which are validated targets in the search for chemicals that synergize with β-lactams. *stk1* and *vraS* knockout (**k/o**) or inactivation enhance[26, 27, 29, 41] the potency of cell wall-acting antibiotics, including β-lactams like oxacillin, against MRSA. **Table 4** shows us that P2As function even when stk1 and vraS are non-functional. Therefore, VraS and Stk1 are not the targets of P2As.

Table 4. Chemical **1** does not function through traditional targets associated with β-lactam resistance. **1** retained activity when the *stk1* and *vraS* genes were knocked out. Therefore, P2As must act through a different mechanism. Either the median MIC value, or the more conservative MIC value, is reported from a minimum of 2 biological replicates. **Supplementary Table S6** shows the complete data.

NTML mutant strain	MIC of Penicillin G (μg/mL)	
	+ 50 μM of P2A **1**	+ DMSO
Δstk1	<0.125, 0.25	2
ΔvraS	0.25	2
ΔsaeS	16	8
ΔarlS	16	16
ΔagrC	8	16
ΔkdpD	8	8
ΔhssS	16	16

ΔnreB	16	8
ΔwalK	8	8
ΔsrrB	<2	8

Even though Stk1 and VraS are not involved, it was possible that other protein kinase-regulated pathways could facilitate penicillin G enhancement. So, we tested other kinase knockouts available in the NTML library. Surprisingly, we found multiple kinase knockouts abrogated the activity of **1**. Many of these pathways facilitate antimicrobial resistance, but many are only known as metabolic regulators or else serve other functions (reviewed[42]) with no apparent connection with β-lactam or other resistance. This observation strongly suggests a global regulator, associated with multiple pathways, must be the target of P2As.

Speculation regarding the target of P2As. GraS, a histidine kinase, is known to regulate several pathways, and is also associated with resistance to β-lactams and other cell wall-acting antimicrobials.[42] While inhibition of GraSR signaling reduces resistance to cefuroxime, which is penicillinase-sensitive, it does not affect oxacillin resistance.[43, 44] This profile fits our observations from **Table 2**, where we have shown an enhancement of penicillinase-sensitive penicillin G, but not oxacillin. Unfortunately, the *graS* and *graR* k/o mutants are unavailable in the NTML library. It would be interesting to model the interactions of GraS with **1**, **2** and **3**, but a BLAST search clearly demonstrated that no appropriate templates are available: the closest structure was WalK from *Bacillus subtilis*, but it had <30% sequence identity to the GraS kinase domain, albeit it was higher in the ATP-binding pocket. The complexity[45] of modeling interactions of chemicals with homology models coupled with absence of experimental confirmation of interaction with GraS and P2As makes it impractical to include those results in this manuscript, but we hope to address this hypothesis in future.

Overall conclusions. We have demonstrated P2As as a class of penicillin G enhancers that function through blaZ suppression, resulting in reduced penicillinase expression. This is an alternate implementation of Moreillon's strategy,[18, 20] switching direct penicillinase inhibitors like clavulanate and sulbactam with a P2A as a *blaZ* suppressor. This is a conceptual innovation. It is critical to note that P2As are already able to reduce the MIC of penicillin G to ≤32 μg/mL. This is at maximum 4-8-fold above the point where PBP2a is unable to prevent penicillins from sterilizing foci of infection (MIC 8 μg/mL).[18-20] The MIC of the MRSA strain used in those experiments was similar to our chosen MRSA (≥128 μg/mL vs. 256 μg/mL), so the results are directly comparable.

Since Augmentin™ is ineffective against MRSA, it is already clear that direct penicillinase inhibitors are not the correct partner to combine with penicillin G. Moreover, using even high concentrations of sulbactam with penicillin G failed to cure all animals infected with MRSA.[19] This is because covalent inhibition of the antimicrobial by penicillinase prevents it from being fully available to act on the intended target – this is a major problem in using direct penicillinase inhibitors. Furthermore, sulbactam induces penicillinase expression.[19] These data clearly demonstrate that blocking penicillinase

expression is a preferable target for enhancement of penicillinase-sensitive β-lactams like penicillin G.

We have presented P2As as a first-in-class agents that suppress penicillinase expression. This is an innovative approach towards developing penicillin G enhancers. Our very early hits are extremely potent, and already virtually eliminate penicillinase, even though they are not quite as potent as recent discoveries such as loratadine[21] that have the same ability. At the same time, loratadine functions via Stk1 – a well-validated target. P2As function differently. VraS, another validated target for β-lactam enhancement, is also not the target. Our very early structure-function study suggests the target is a single entity, although we cannot discount the possibility that similar binding pockets across more than one target could also exist. On the other hand, it seems P2As act at a global regulator either upstream or downstream of multiple signal transduction pathways, as multiple kinase knockouts abrogated their activity – this, and the parallel retention of activity against *Δstk1*, *ΔvraS* and *ΔsrrB* strains, suggests this is not non-specific toxicity.

P2As are therefore a promising avenue for drug discovery. A full structure-function study and target identification will help establish these matters further – these are our goals for the future.

MATERIALS AND METHODS

Sources of bacteria, chemicals and reagents. All the chemicals tested for synergy with penicillin G (**Table 1**) were purchased from ChemBridge. Purity was standard, at 85-100% based on HPLC profiles. All standard chemicals and reagents were purchased from Sigma Aldrich and/or Fisher Scientific. All bacterial strains used in this study are reported in **Table 5**.

Table 5. Strains used in this study.

Strain	Description	Source
S. aureus		
USA300 MRSA (ATCC BA-1717)	Community-acquired MRSA strain	ATCC
SAUSA300_1113	Transposon mutant Δ*stk1* NE217	NARSA
SAUSA300_2035	Transposon mutant Δ*kdpD* NE434	NARSA
SAUSA300_2309	Transposon mutant NE820	NARSA
SAUSA300_2338	Transposon Mutant NE1157	NARSA
SAUSA300_1441	Transposon Mutant Δ*srrB* NE588	NARSA
SAUSA300_1638	Transposon Mutant Δ*phoR* NE618	NARSA
SAUSA300_1866	Transposon Mutant Δ*vraS* NE823	NARSA
SAUSA300_0690	Transposon Mutant Δ*saeS* NE1296	NARSA
SAUSA300_1307	Transposon Mutant Δ*arlS* NE1183	NARSA
SAUSA300_1991	Transposon Mutant Δ*agrC* NE873	NARSA
Mu50 Rosenbach VISA (ATCC 700699)	Vancomycin Intermediate-Resistant *S. aureus*	ATCC

E. coli (ATCC 35128)	β-lactamase producing *E. coli* quality control strain	ATCC
E. coli (ATCC 25922)	Non-β-lactamase producing *E. coli* quality control strain	ATCC

Antimicrobial susceptibility testing. MIC assays were run as per CLSI guidelines, by incubating $5e^5$ CFU/mL bacteria with or without antibiotic in cation-adjusted Müller-Hinton Broth (CA-MHB). The concentration of bacteria was confirmed by serial dilution and plating on Tryptic Soy Agar (TSA). Antibiotic concentrations were confirmed by testing the MIC of quality control strains. A serial dilution of antimicrobial was first generated, and bacterial culture was then added to it: Each well in a 96-well plate contained 200 µL total volume, comprising 100 µL of bacterial culture at ~$1e^6$ CFU/mL and 100 µL of antimicrobial or chemical at 2X concentration. These plates were incubated at 37 °C for 18 hours and observed visually for signs of growth, and then confirmed using a microplate reader at wavelength of 600 nm.

MRSA growing without antibiotic served as the positive control for growth, while uninoculated CA-MHB served as negative control. We ensured MRSA status by testing the MIC of vancomycin was ≤2 µg/mL and MIC of oxacillin was >2 µg/mL, in addition to confirming the expression of PBP2a. In comparison, Escherichia coli strains ATCC 35218 and ATCC 25922 showed no PBP2a expression, and were unaffected by vancomycin.

When testing for potency enhancement of antimicrobials, the MIC procedure was modified to include 100 µL of a mixture containing the antimicrobial and the chemical to be tested, both at 2X concentration, to replace 100 µl of the antimicrobial alone. The remaining procedures remained unaltered.

Nitrocefin assay with live MRSA. MRSA growth curves were constructed to identify the log phase (5-7 hours of incubation at 37 °C after 1:1,000 dilution of an overnight culture yielded exponential growth). Bacteria were grown under different conditions (with or without antimicrobials/chemicals) for 7 hours. For uninduced samples, ~$5e^5$ CFU/mL of MRSA was incubated with CA-MHB and one of chemical 1, 2, or 3 at a final concentration of 50 µM. A v:v equivalent DMSO was used as control. For samples induced to produce penicillinase, ~$5e^5$ CFU/mL of MRSA was incubated with 64 µg/mL penicillin G in CA-MHB and chemical at a concentration of 50 µM. Again, DMSO was used as control. Solutions were incubated at 37 ^{o}C for 7 hours and centrifuged at 2500 rpm. Pellets were resuspended in PBS at a pH of 7 and OD_{600} was recorded to compare MRSA growth. Supernatant fluid was incubated with nitrocefin at a concentration of 500 µg/mL for 45 minutes and color changes were analyzed via ImageJ software.

Nitrocefin assay with purified β-lactamase. We compared nitrocefin hydrolysis of purified β-lactamase blend (Sigma, catalog # L7920) in the presence of chemicals 1, 2, and 3. Pure β-lactamase at a concentration of 1 mg/mL was serially diluted. Each well was then combined with either DMSO or chemical (**1**, **2**, or **3**). Nitrocefin was added at a concentration of 500 µg/mL and the solutions were incubated at room temperature for 2-3 minutes. Color changes were recorded at 486 nm to determine any direct inhibitory

effect of these chemicals on purified β-lactamase. The data reported in this manuscript corresponds to the lowest concentration we tested, to ensure maximum opportunity for inhibition by the chemicals.

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