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β-Lactamase Suppression as a Strategy to Target Methicillin-Resistant *Staphylococcus aureus*: Proof of Concept

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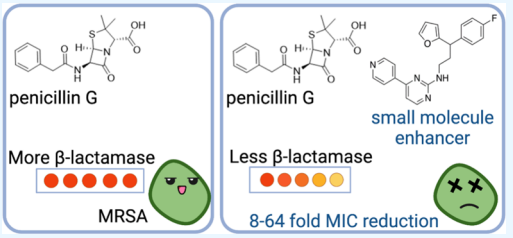
ABSTRACT: β-Lactamase (penicillinase) renders early, natural β-lactams like penicillin G useless against methicillin-resistant *Staphylococcus aureus* (MRSA), which also expresses PBP2a, responsible for resistance to semisynthetic, penicillinase-insensitive β-lactams like oxacillin. Antimicrobial discovery is difficult, and resistance exists against most treatment options. Enhancing β-lactams against MRSA would revive its clinical utility. Most research on antimicrobial enhancement against MRSA focuses on oxacillin due to β-lactamase expression. Yet, Moreillon and others have demonstrated that penicillin G is as potent against a β-lactamase gene knockout strain, as vancomycin is against wild-type MRSA. Penicillin G overcame PBP2a because β-lactamase activity was blocked. Additionally, animals treated with a combination of direct β-lactamase inhibitors like sulbactam and clavulanate with penicillin G developed resistant infections, clearly demonstrating that direct inhibition of β-lactamase is not a good strategy. Here, we show that 50 μM pyrimidine-2-amines (P2As) reduce the minimum inhibitory concentration (MIC) of penicillin G against MRSA strains by up to 16-fold by reducing β-lactamase activity but not by direct inhibition of the enzyme. Oxacillin was not enhanced due to PBP2a expression, demonstrating the advantage of penicillin G over penicillinase-insensitive β-lactams. P2As modulate an unknown global regulator but not established antimicrobial-enhancement targets Stk1 and VraS. P2As are a practical implementation of Moreillon's principle of suppressing β-lactamase activity to make penicillin G useful against MRSA, without employing direct enzyme inhibitors.



INTRODUCTION

Antimicrobial resistance (AMR) has emerged in *Staphylococcus aureus* stepwise.[1] The introduction of penicillins such as penicillin G in the 1940s led to penicillinase-based resistance via *blaZ* and related genes. *blaZ* encodes penicillinase. Staphylococcal penicillinase is secreted outside the cell and destroys sensitive β-lactams before they can act upon the pathogen. Upon the introduction of penicillins that are resistant to penicillinases—such as methicillin and oxacillin—methicillin-susceptible strains (MSSA) gave way to MRSA when the bacterium acquired *mecA*. MRSA expresses both penicillin-binding protein 2a (PBP2a) as well as penicillinases.[1,2] MRSA is, 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] are emerging against these and other treatment options. Resistance will only increase with antimicrobial use.[7]

Community- and hospital-acquired *S. aureus* infections are common today.[8] 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.[9] 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, nontraditional options are also being considered. Identifying enhancers[10] (chemicals that increase the potency of clinically relevant antimicrobials) has already resulted in approved, marketed drugs such as Augmentin,[11] 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, which is widely expected[12] to overcome β-lactams. On the other hand, Moreillon and others have put

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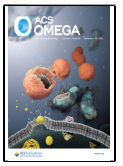

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forward strong evidence[13,14] that older, natural penicillins and aminopenicillins are more effective against MRSA than semisynthetic penicillins such as oxacillin, if penicillinase is not present. The absence of penicillinase allowed a higher concentration of antibiotic to become available, leading to powerful inhibition of both cell wall-synthesizing enzymes, PBP2 and PBP2a. This helped cure the infection. This is a major advantage over β-lactams like oxacillin and methicillin, which are unable to overcome PBP2a. They also found that these penicillins sterilize infective endocarditis vegetations in rabbits within 4 days of therapy if penicillinase was ineffective. Penicillin G was comparable to vancomycin in an animal model when penicillinase was not present;[15] Penicillin G cured just as many rabbits infected with penicillinase-negative MRSA as vancomycin did against wild-type MRSA. Penicillin G was actually more potent than vancomycin against the penicillinase negative MRSA strain (Log_{10}CFU/gram of infected tissue: ~4.6 ± 2 vs ~7 ± 1 after treatment, respectively).[15] 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 in treatment. It is evident that 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.[14] As a result, the pathogen is able to survive. Notably, penicillinase knockout strains did not demonstrate similar failures or resistance. In light of the above, our hypothesis is that inhibiting β-lactamase activity (potentially by inhibition of expression or secretion) 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[16] could help revive the clinical utility of natural penicillins, which are currently considered of little to no clinical utility against this pathogen.

Here, we report the discovery of P2As as a novel class of chemical enhancers that resensitize MRSA to penicillin G by up to 16-fold by suppression of β-lactamase activity. The structure of the prototype P2A is shown in Figure 1. It was

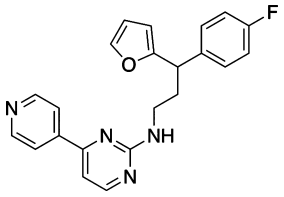

Figure 1. Structure of the prototype P2A (**1**) that enhances the potency of penicillin G against USA300 MRSA strain ATCC BAA-1717. The chemical was in its racemic form, as were all chiral center-containing structural analogues tested during this study. We are unable to identify which enantiomer is responsible for activity at this time.

discovered in a phenotypic screen for penicillin G enhancement (see Materials and Methods). 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.

Our chemicals resensitize MRSA to penicillin G but not penicillinase-resistant antimicrobials such as oxacillin. Recent literature reports[17−19] on enhancer discovery have focused on two central pathways controlling oxacillin resistance: A eukaryotic-like serine/threonine kinase called Stk1[20−22] and a histidine kinase called VraS.[23,24] 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 Table S1). The MIC of penicillin G was 256 μg/mL (range: 128 μg/mL to >256 μg/mL, with the mode being 256 μg/mL), but addition of 50 μM chemical **1** enhanced the potency by 16-fold. The MIC of penicillin G without chemicals or DMSO present was also 256 μg/mL. A titration showed that at least 50 μM P2As was necessary to induce ≥4-fold MIC reduction for penicillin G, so the effect is additive, not synergistic. This is understandable, as these are only early hits, and will require significant optimization. Our goal is only to reveal the substantially different mechanism of penicillin G enhancers (see details below) in comparison with contemporary[17−19,25] literature.

Recent studies have reported enhancement of oxacillin potency against MRSA strains by inhibition of Stk1 and VraS.[17−19] 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. However, other penicillinase-sensitive antibiotics, amoxicillin and ampicillin, show a 4-fold enhancement in MIC against the same strain of bacteria, as can be expected from the proposed penicillinase suppression. The MIC was 256 μg/mL for both amoxicillin and ampicillin, and in the presence of 50 μM of chemical **1**, it fell to 64 μg/mL.

Based on the above, we hypothesized that P2As function by either directly or indirectly inhibiting MRSA penicillinases. Table 3 demonstrates that penicillinase activity of live MRSA in a nitrocefin assay was indeed reduced. Loratadine, the positive control,[17] was clearly the best suppressor of penicillinase. **1** showed a slightly reduced suppression in comparison, while **2** and **3** were significantly less potent. Additional details can be seen in Table S3. So clearly, these chemicals reduce penicillinase activity in MRSA. At the same time, a control experiment showed that **1** did not inhibit penicillinase secreted by MRSA where live cells were removed using a 22 μm filter (Table 4). Rate of hydrolysis was measured for two biological replicates (penicillinase extracted from two separate MRSA cultures). The rate of hydrolysis in the presence of sulbactam, a known direct penicillinase inhibitor, was marked as 0%, and DMSO as 100%. Clearly, nitrocefin hydrolysis was minimum when sulbactam was present, while chemicals **1**, **2**, and **3** showed at least as much nitrocefin hydrolysis as the negative controls. This conclusively demonstrates P2As function by suppressing penicillinase

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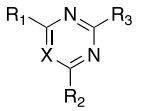
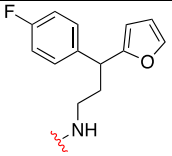
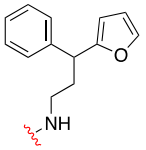
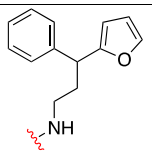
Table 1. Changes in Chemical Structure Demonstrates Varied Ability to Enhance Penicillin G Potency against USA300 MRSA ATCC BAA-1717[a]



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					16	16
2					32	8
3					32	8
4 to 15	Various changes (see **Fig 2** and **Table S1**)				128 to >256	≤2

[a]At least 2 biological replicates were run for all chemicals; 3 replicates were run for chemicals **1**−**3**. MICs reported below represent the median. Complete data for all replicates is reported in Table S1.

Table 2. Effect of the Prototype P2A (1) on Penicillinase-Sensitive/Resistant β-Lactams and Other Antimicrobials[a]

	MIC (μg/mL)	
antibiotic	+DMSO	+50 μM P2A 1
oxacillin	128	256
chloramphenicol	32	32
vancomycin	1	1
amoxicillin	256	64
ampicillin	256	64

[a]Biological replicates were performed; the median value is reported. Further details can be found in Table S2.

Table 3. Nitrocefin Assay Demonstrates P2As Reduce Penicillinase Activity of MRSA Strain ATCC BAA-1717[a]

chemical @ 50 μM	% β-lactamase activity when compared with DMSO control
Loratadine	19 ± 7
1	24 ± 21
2	71 ± 3
3	79 ± 7

[a]See Materials and Methods and Table S4 for details. Error is reported as standard deviation, based on two biological replicates.

activity of live cells and not by direct neutralization of the enzyme. This is potentially due to reduced penicillinase expression. There is a slim chance that penicillinase secretion is being blocked, but dissection of such nuanced details is unnecessary at this time. Instead, our focus is to demonstrate that reducing hydrolysis of penicillin G using chemicals helps overcome not only PBP, the usual target of β-lactams, but also PBP2a-based resistance mechanisms. This is now unequivocally established.

Table 4. P2As Are Unable to Block Nitrocefin Hydrolysis by Penicillinase[a]

	1	2	3	sulbactam	DMSO
% nitrocefin hydrolysis rate	219.27	129.36	124.77	0.00	100.00

[a]See Materials and Methods section for details.

P2As Bind a Hypothetical, Tight Pocket. We have performed structure activity work to explore the space around the P2A scaffold. While Table 1 demonstrates the chemicals which were able to enhance penicillin G potency against MRSA, Figure 2 identifies various structural variants that failed to do so. The limited number of structural variants that retain the penicillin G enhancement phenotype strongly suggest that they bind a very well-defined pocket. This hypothetical pocket has been envisioned in Figure 3.

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** and **2** in having no substitution at R1

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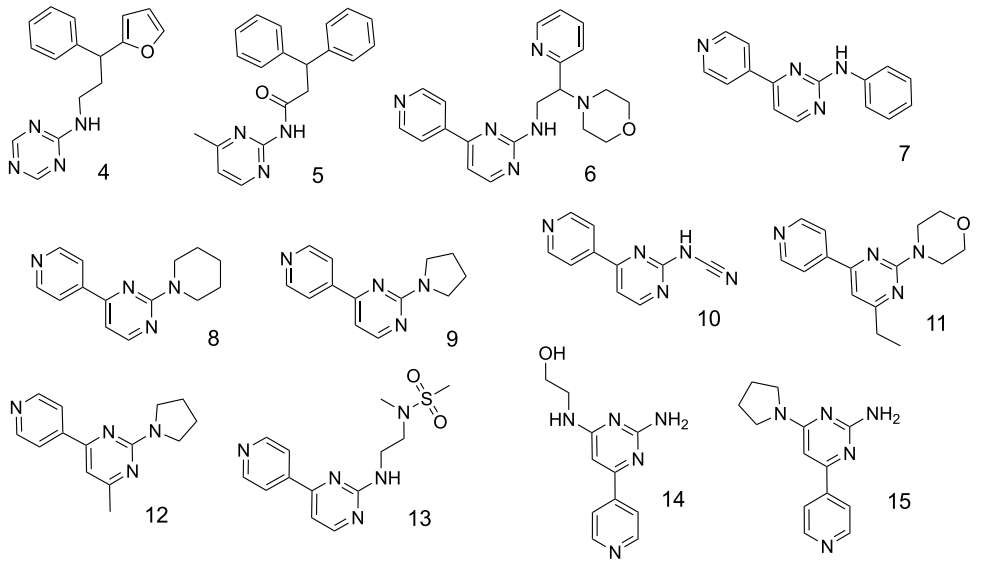

Figure 2. Structural variants of **1** that fail to enhance penicillin G activity against MRSA. A selection of chemicals containing the P2A core scaffold were tested for their ability to enhance penicillin G but failed to show any discernible activity.

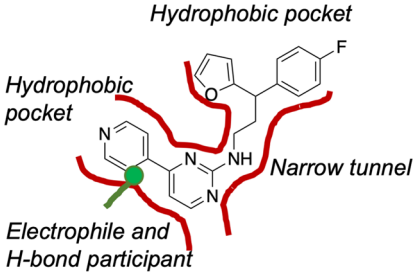

Figure 3. Hypothetical binding pocket for P2As. The P2A core binds in the main pocket, while the R1 substituent binds in a narrow groove possessing an electrophile. The nature of the R2 group-binding region is uncertain, but small groups have been tolerated well. In comparison, our observations suggest the R3 groups bind in an elongated, narrow tunnel that opens up to a wider groove.

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 rather 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 sp^3 carbon and nitrogen, but still lacks penicillin-enhancement activity, perhaps because it would not fit into this tunnel due to its rigid, linear nature.

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, it is a combination of both factors. **13**, combined with **5**, seems 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 flexible 2-hydroxy-ethyl-1-amino- and 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 R3 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

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complete loss of activity. Therefore, we have reported further characterization only for these chemicals.

P2As Enhance Penicillin G against Multiple MRSA Strains. We investigated the ability of P2As to reduce MIC of penicillin G against additional MRSA strains. We chose to test several PFGE variants available through ATCC (see Materials and Methods) in the same manner as with strain BAA-1717, because these represent a large selection of causative MRSA agents in human diseases. These are all β-lactamase positive strains (confirmed using nitrocefin), and hence relevant to the question at hand. The altered susceptibility data is reported in Table 5. Addition of P2As enhanced the potency of penicillin G by as much as 32-fold in these strains. It is therefore reasonable to expect that chemicals optimized to suppress beta-lactamase expression in MRSA will help enhance β-lactamase susceptible penicillins to treat a wide range of infections.

Table 5. P2As also Enhance Penicillin G against Multiple Clinically Relevant MRSA Strains[a]

	fold reduction in MIC (μg/mL) of penicillin G in the presence of 50 μM chemical **1**, **2**, or **3**		
MRSA strain	**1**	**2**	**3**
USA 100	4−8	8	4−8
USA 200	16−32	2−4	2−4
USA 400	2−8	8−16	8−16
USA 500	4	4−8	2−4

[a]Two biological replicates were performed. The full range is presented where variability was observed. Importantly, all MRSA strains showed increased susceptibility to penicillin G in the presence of P2As.

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[17,18,20,22,26] 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[21,22,24,27] the potency of cell wall-acting antibiotics, including β-lactams like oxacillin, against MRSA. Table 6 shows us that P2As function even when stk1 and vraS are nonfunctional. Therefore, VraS and Stk1 are not the targets of P2As.

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[28]) 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.[28] While inhibition of GraSR signaling reduces resistance to cefuroxime, which is penicillinase-sensitive, it does not affect oxacillin resistance.[29] 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[30] of modeling interactions of chemicals with homology models coupled with the absence of experimental confirmation of interaction with GraS and P2As makes it impractical to include those results in this manuscript.

Table 6. Chemical 1 Does Not Function through Traditional Targets Associated with β-Lactam Resistance[a]

	MIC of Penicillin G (μg/mL)	
NTML mutant strain	+50 μM of P2A 1	+DMSO
Δ*stk1*	<0.125, 0.25	2
Δ*vraS*	0.25	2
Δ*saeS*	16	8
Δ*arlS*	16	16
Δ*agrC*	8	16
Δ*kdpD*	8	8
Δ*hssS*	16	16
Δ*nreB*	16	8
Δ*phoR*	8	8
Δ*srrB*	<2	8

[a]**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. Table S4 shows the complete data.

Overall Conclusions. We have demonstrated P2As as a class of penicillin G enhancers that function through *blaZ* suppression, resulting in reduced penicillinase activity. This is an alternate implementation of Moreillon's strategy,[14,16] 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).[14−16] 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.[15] This is because hydrolysis 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.[15] 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 first-in-class agents that suppress penicillinase activity without direct inhibition of the enzyme. This is an innovative approach toward 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[17] that have

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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 Δ*stk1*, Δ*vraS*, and Δ*srrB* strains, suggests this is not nonspecific toxicity.

P2As are therefore a promising avenue for drug discovery. The fact that they function differently when compared to direct β-lactamase inhibitors makes them a practical implementation of Moreillon's principle.

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 95−100% based on LC-MS profiles (Figures S1−S3 for **1**−**3**). 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 7.

Table 7. Strains Used in This Study

strain	description	source
USA300 MRSA (ATCC BA-1717)	Community-acquired USA 300 MRSA strain	ATCC
ATCC BAA-1761	USA 100 MRSA strain	ATCC
ATCC BAA-1720	USA 200 MRSA strain	ATCC
ATCC BAA-1707	USA 400 MRSA strain	ATCC
ATCC BAA-1763	USA 500 MRSA strain	ATCC
SAUSA300_1113	Transposon mutant Δ*stk1* NE217	NARSA[a]
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

[a]Network on Antimicrobial Resistance in *Staphylococcus aureus* (NARSA) for distribution by BEI Resources.

Antimicrobial Susceptibility Testing. MIC assays were run as per Clinical and Laboratory Standards Institute (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 ∼5e^5 CFU/mL and 100 μL of antimicrobial or chemical at 2× concentration. These plates were incubated at 37 °C for 18 h, observed visually for signs of growth, and then confirmed using a microplate reader at a wavelength of 600 nm.

MRSA growing without antibiotic served as the positive control for growth, while uninoculated CA-MHB served as the negative control. We ensured MRSA status by ensuring the MIC of vancomycin was ≤2 μg/mL and MIC of oxacillin was >2 μg/mL, as defined by the CLSI.[31] Additionally, we have also performed the Abbott Laboratories ClearView test to confirm the presence of PBP2a in our MRSA strain as a secondary precaution. In comparison, *Escherichia coli* strains ATCC 35218 and ATCC 25922 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.

All MIC tests were performed at least 2 times, using separate overnights grown from distinct colonies.

Single-Point Screen to Identify Enhancers. The 5e^5 CFU/mL USA300 MRSA strain ATCC BAA-1717 was incubated with 1/4*MIC of penicillin G, with either 50 μM chemical (dissolved in 100% DMSO) or else DMSO as a negative control. The bacterial culture concentration was ensured by serial dilution and CFU counts.

Nitrocefin Assay with Live MRSA. MRSA growth curves were constructed to identify the log phase (5−7 h of incubation at 37 °C after 1:1000 dilution of an overnight culture yielded exponential growth). Bacteria were grown under different conditions (with or without antimicrobials/chemicals) for 7 h. 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 of DMSO was used as control, amounting to a final proportion of 0.5%. 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 a control. Solutions were incubated at 37 °C for 7 h and centrifuged at 2500 rpm. Supernatant fluid was incubated with nitrocefin at a concentration of 500 μg/mL for 45 min, and color changes were analyzed via ImageJ software. Each experiment was repeated one more time. Pellets were resuspended in PBS at a pH of 7 and OD_{600} was recorded to compare MRSA growth.

Nitrocefin Assay with Secreted β-Lactamase. For the experiment with penicillinase secreted by our MRSA samples, we obtained the enzyme by filtering an overnight culture through a 0.22 μm filter to eliminate all bacterial cells. We compared nitrocefin hydrolysis of secreted penicillinase in the presence of chemicals **1**, **2**, and **3** or an equal concentration (50 μM) of sulbactam. Penicillinase obtained above was incubated with sulbactam or our chemicals for 2 min. Nitrocefin was added at a concentration of 500 μg/mL. A v:v equivalent DMSO concentration (0.5%) was used as control. Two independent samples (secreted penicillinase from two overnight cultures, grown from separate MRSA colonies) were run for each condition. Absorbance (486 nm) was

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monitored for 23 min (a lag period of 7 min was eliminated during analysis). The absorbance changes were converted to a percent scale with DMSO (negative control) at 100% and sulbactam (positive control) at 0% penicillinase activity.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsomega.2c04381.

Detailed structure−activity tables for P2As, reporting the complete MIC data for penicillin G, biological replicates for MIC of various antimicrobials in the presence and absence of chemicals, β-lactamase expression levels in the presence of P2As relative to negative control, MIC changes for penicillin G in the presence and absence of chemicals, data on kinetic analysis of penicillinase-based nitrocefin hydrolysis in the presence and absence of chemicals, and chemical characterization data for all reported chemicals (PDF)

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Author Contributions

Conceptualization: A.S.; Methodology: P.M.T., M.A.D., and A.S.; Data collection: P.M.T. and M.A.D.; Formal analysis: P.M.T. and A.S.; Writing and editing: P.M.T. and A.S. All authors have read and agreed to the published version of this manuscript.

Notes

The authors declare no competing financial interest.

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