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GW779439X and Its Pyrazolopyridazine Derivatives Inhibit the Serine/Threonine Kinase Stk1 and Act As Antibiotic Adjuvants against β -Lactam-Resistant *Staphylococcus aureus*

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Abstract

As antibiotic resistance rises, there is a need for strategies such as antibiotic adjuvants to conserve already-established antibiotics. A family of bacterial kinases known as the penicillin-binding-protein and serine/threonine kinase-associated (PASTA) kinases has attracted attention as targets for antibiotic adjuvants for β -lactams. Here, we report that the pyrazolopyridazine GW779439X sensitizes methicillin-resistant *Staphylococcus aureus* (MRSA) to various β -lactams through inhibition of the PASTA kinase Stk1. GW779439X potentiates β -lactam activity against multiple MRSA and MSSA isolates, including the sensitization of a ceftaroline-resistant isolate to ceftaroline. *In silico* modeling was used to guide the synthesis of GW779439X derivatives. The presence and orientation of GW779439X's methylpiperazine moiety was crucial for robust

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Author Contributions

A.S. performed experiments, analyzed results, and wrote the manuscript. N.W. performed experiments and analyzed results. D.D. and C.F. designed/performed chemical synthesis and was involved in study design, data analysis, and manuscript preparation. W.Z., W.E.R., R.S., and J.D.S. were involved in study design, data analysis, and manuscript preparation. All authors edited the manuscript.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acsinfecdis.8b00136.

Complete list of kinase inhibitor library percent inhibition values (XLSX)

Structures of validated kinase inhibitor hits from kinase inhibitor library screen (Figure S1); GW779439X bacterial toxicity data (Figure S2); dose–response curves of SB-202190 (Figure S3); *in silico* docking models of pyrazolopyridazine analogs (Figure S4); bacterial strains used in this study (Table S1) (PDF)

¹H and ¹³C NMR validation of synthesized compounds (PDF)

The authors declare no competing financial interest.

biochemical and microbiologic activity. Taken together, our data provide a proof of concept for developing the pyrazolopyridazines as selective Stk1 inhibitors which act across *S. aureus* isolates.

Graphical Abstract

Keywords

MRSA; PASTA kinase; β -lactam; drug discovery; pyrazolopyridazine; antibiotic

The β -lactams are one of the oldest and most successful classes of antibiotics; however, they are also arguably the most affected by antibiotic resistance. Methicillin-resistant *Staphylococcus aureus* (MRSA) quickly evolved as a form of β -lactam resistance, and it continues to be a relevant threat in the clinic and the community.¹ Unfortunately, resistance to front-line MRSA treatments such as vancomycin, daptomycin, ceftaroline, and linezolid has begun to appear in the clinic,^{2–5} highlighting the need for novel antibiotic strategies. One such strategy is the utilization of antibiotic adjuvants to preserve the efficacy of current antibiotics.⁶ This strategy has already proven successful in the case of β -lactam/ β -lactamase inhibitor combination therapy to treat various β -lactamase-expressing Gram-negative infections and penicillin-resistant *S. aureus*.⁷ However, because MRSA strains utilize the intrinsically β -lactam resistant penicillin-binding protein PBP2A as their primary resistance mechanism, alternative adjuvants are needed to augment β -lactams in these strains.

Over the past decade, there has been an increased interest in exploring bacterial signal transduction mechanisms, in particular those regulated by protein kinases, as potential antibiotic and antivirulence targets.^{8,9} While prokaryotic protein kinases were originally thought to only phosphorylate proteins on histidine and aspartate residues,^{10,11} it is now well-established that eukaryotic-like serine/threonine protein kinases (eSTKs) are near ubiquitous in bacteria.¹² Of note, a subset of eSTK known as the penicillin-binding protein and Ser/Thr kinase-associated (PASTA) kinases has been demonstrated to be crucial for cell wall homeostasis,^{13,14,23,15–22} virulence,^{9,23,24} biofilm formation,²⁵ germination,^{26,27} and metabolism^{28,29} in a variety of *Firmicutes* and *Actinobacteria*. Intriguingly, while the PASTA kinase PknB is essential for the viability of *Mycobacterium tuberculosis*,^{30,31}

deletion of PknB homologues in the *Firmicutes* decreases resistance to the β -lactam antibiotics (reviewed in ref 9). This characteristic makes the PASTA kinases attractive targets for potential β -lactam adjuvants.

Eukaryotic protein kinases have been extensively studied and successfully exploited in the clinic as therapeutic targets for anticancer treatments.³² As such, there is established knowledge and resources such as small-molecule protein kinase inhibitor libraries that can be repurposed for the identification of eSTK-targeting scaffolds. We and others have published multiple PASTA kinase inhibitors.^{20,22,33–37} Here, we report that GW779439X, a pyrazolopyridazine identified in a small-molecule kinase inhibitor library screen, increases the sensitivity of the MRSA strain LAC to β -lactam antibiotics via inhibition of the PASTA kinase Stk1. In addition, GW779439X also sensitizes a variety of other MRSA and further enhances β -lactam activity against methicillin-sensitive *S. aureus* (MSSA) isolates in a similar fashion. Finally, using a series of GW779439X congeners, we propose a preliminary structure–activity relationship (SAR) for the pyrazolopyridazine pharmacophore's activity against the Stk1 kinase domain. Taken together, our data demonstrate that GW779439X and the pyrazolopyridazines are promising lead compounds for further optimization as Stk1 inhibitors as adjuvants to β -lactams for certain MRSA strains.

RESULTS AND DISCUSSION

GW779439X Sensitizes MRSA to β -Lactams.

In a variety of Gram-positive pathogens, including *S. aureus*, genetic deletion of the PASTA kinases results in increased sensitivity to β -lactam antibiotics.^{13,18,20,23} Through a kinase inhibitor library screen, we previously identified the compound GSK690693 as a potent inhibitor of the *Listeria monocytogenes* PASTA kinase PrkA which sensitizes the organism to β -lactams.²² However, GSK690693 showed only minor biochemical activity against the *S. aureus* PASTA kinase Stk1 and no appreciable activity microbiologically as a β -lactam adjuvant against *S. aureus*. Therefore, to identify inhibitors of Stk1 that could function as β -lactam adjuvants, we screened 1147 small molecule kinase inhibitors from the GlaxoSmithKline Published Kinase Inhibitor Sets 1 and 2 (PKIS1 and PKIS2)^{38,39} and Selleck kinase inhibitor libraries against the wild-type MRSA strain LAC in the presence of a sublethal dose of ceftriaxone. Thirty-one compounds potentiated ceftriaxone activity three standard deviations or greater than the library average (Figure 1A). Of these, 25 compounds failed to show a dose response in secondary screens, and an additional 3 demonstrated β -lactam independence. The three remaining compounds were NVP-ADW742, BI-2536, and GW779439X (Supplemental Figure S1). Of these, we selected GW779439X (Figure 1A, cyan dot, and B) for further analysis both because it was the most potent compound in our secondary screens and due to the availability of molecular derivatives (Figure 1A, red dots).

Genetic deletion of *pknB* (which expresses Stk1 and will be referred to as *stk1* throughout this manuscript) has been shown to decrease resistance of MRSA to a variety of β -lactams.^{13,23,35} We therefore hypothesized that if GW779439X acts through an Stk1-dependent mechanism, it should be able to potentiate other β -lactams beyond ceftriaxone. To test this hypothesis, we determined the minimum inhibitory concentration (MIC) values of a variety of antibiotics against LAC in the presence and absence of 5 μ M GW779439X. Importantly,

GW779439X has no intrinsic effects on bacterial growth below 20 μM (Supplemental Figure S2). As can be seen in Table 1, GW779439X was able to potentiate the activity of all β -lactams tested, particularly the penicillinase-resistant penicillins oxacillin and nafcillin to an MIC considered susceptible to these agents. Intriguingly, GW779439X also increased sensitivity twofold to ceftaroline, one of the few clinically available β -lactams with anti-MRSA activity. Importantly, GW779439X was unable to further sensitize a *stk1* mutant to any of the β -lactams tested. Consistent with the PASTA kinase literature, the MICs of non- β -lactams such as vancomycin or chloramphenicol remained unaffected in any condition tested. Taken together, these data support the hypothesis that GW779439X potentiates a variety of β -lactam antibiotics in an Stk1-dependent manner and shifts the β -lactam class collectively toward sensitivity.

GW779439X Biochemically Inhibits Stk1 *in Vitro*

Because GW779439X was originally designed to be a eukaryotic kinase inhibitor⁴⁰ and its effects on β -lactam MICs are Stk1-dependent, we hypothesized that GW779439X acts through direct inhibition of the Stk1 kinase domain. Using autoradiography, we monitored the *in vitro* kinase activity of the purified Stk1 kinase domain in the presence of increasing concentrations of GW779439X. Strikingly, we observed a robust inhibition of Stk1 autophosphorylation and phosphorylation of the non-specific phosphosubstrate myelin basic protein (MBP) at concentrations of GW779439X as low as 2 μM (Figure 2). This is in contrast to the compound SB-747651A, a compound which failed to sensitize LAC to ceftazidime in our library screen and which we've previously demonstrated has no intrinsic activity against the *L. monocytogenes* PASTA kinase PrkA (Figure 2).²² When taken with our broth culture experiments, these data support the hypothesis that GW779439X potentiates β -lactam activity via direct inhibition of Stk1.

GW779439X Potentiates Oxacillin Activity against Various *S. aureus* Strains.

LAC was chosen as the MRSA strain for our primary screen for multiple reasons. It is a representative of the USA300 clonal lineage, and it is a particularly virulent and prevalent pulsed-field gel electrophoresis (PFGE) type among community-acquired MRSA infections in the United States.⁴¹ To determine if GW779439X would be effective against additional clonal lineages, we determined the MIC of oxacillin against a variety of MRSA and MSSA isolates in the presence and absence of GW779439X (Table 2). At 5 μM GW779439X, we observed potentiation of oxacillin against representatives of PFGE types USA400, USA600, and USA800 as well as the hospital-acquired MRSA strain COL. Strikingly, GW779439X lowered the MIC at least 512-fold against ATCC BAA-2686, a MRSA isolate isolated from the blood of a patient with cystic fibrosis which evolved high-level resistance to ceftaroline; this phenotype was also observed when tested with ceftaroline (Figure 3A) in a ceftaroline-dependent manner (Figure 3B). Finally, GW779439X was also able to modestly potentiate oxacillin activity against the MSSA isolates Newman and NCTC8325 (Table 2). These data demonstrate that a broad range of *S. aureus* isolates are susceptible to β -lactam potentiation by GW779439X, and combination therapy with kinase inhibitors may represent a particularly powerful therapeutic option in the context of ceftaroline-resistant *S. aureus*.

Subset of Pyrazolopyridazines Retain Biochemical and Microbiologic Activity.

Because GW779439X was originally designed to target human CDK4, its direct utility as an antibiotic adjuvant is limited. To rationally design modifications which would minimize off-target effects, a better understanding of the GW779439X-Stk1 SAR is needed. To focus our efforts, we utilized the program Autodock4.2 to dock GW779439X into the active site of the Stk1 kinase domain (Figure 4A). GW779439X's core scaffold consistently docks into the Stk1 active site in a fashion similar to that of other pyrazolopyridazines in CDK2⁴⁰ with the aminopyrimidine moiety participating in a hydrogen bonding pattern with the backbone atoms of Ile90. The pyrazolopyridazine head pivots almost perpendicular to the active site floor, allowing for a potential parallel-displaced π -stacking interaction with Phe150. The *p*-*N*-methyl piperazine tail reaches out of the active site and appears to anchor to Glu97 via salt bridge. Finally, the *m*-trifluoromethyl group comes to rest against Thr94. This model gave us an initial reference for selecting chemical modifications of GW779439X.

In our primary kinase inhibitor library screen (Figure 1), the pyrazolopyridazine family members as a group possessed modifications at four locations (Figure 4B). Based on our *in silico* model, we speculated that addition of side chains at positions R2 and R4 would not be tolerated, so we chose to leave these positions unmodified. We retained the pyridazine nitrogen at the R3 position to remain consistent with GW779439X. Because we hypothesized the *p*-*N*-methyl piperazine is important for a stabilizing salt bridge with Glu97, we restricted the R1 position to the original aniline and allowed for modifications on this moiety at an R5 and R6 position which would come within proximity of Glu97 (Figure 4C). Utilizing Scheme 1, we ultimately synthesized seven compounds with R5 and R6 modifications to test both biochemically and micro-biologically (Table 3).

Using autoradiography, we first tested the ability of our compound series to inhibit Stk1 activity *in vitro*. At an inhibitor concentration of 2 μ M, the compounds CAF052 and CAF078 robustly inhibited Stk1 kinase activity like the GW779439X control (Figure 5A). In contrast, CAF070, CAF075, and CAF089 possessed very little activity at this concentration. The final two compounds, CAF045 and CAF077, displayed an intermediate phenotype with partial Stk1 inhibition. From these data, we hypothesized that the inhibition profile of our compound series would directly translate to the potentiation of oxacillin in broth culture. When tested against LAC with a sublethal concentration of oxacillin and an inhibitor concentration of 20 μ M, the compounds CAF045, CAF052, CAF077, and CAF078 mimicked the GW779439X control with a near 100% inhibition of growth relative to bacteria treated with oxacillin alone (Figure 5B). In agreement with our autoradiography data, the compounds CAF070, CAF075, and CAF089 were significantly less effective at potentiating oxacillin in broth culture. To determine the potencies of the four most-efficacious compounds relative to GW779439X, we treated LAC with a sublethal dose of oxacillin and decreasing concentrations of the kinase inhibitors. We discovered that CAF078 had a potency similar to GW779439X, achieving complete inhibition of bacterial growth at submicromolar concentrations (Figure 5C). On the other hand, CAF052 was 4-fold less potent than GW779439X in broth culture. CAF045 and CAF077 were both 32-fold less potent than GW779439X with near-complete inhibition of growth occurring only as low as 10 μ M. Taken together, our data demonstrate that multiple family members of the

pyrazolopyridazine class of compounds retain both biochemical and microbiologic activity, though with a wide variation in potency. Importantly, our data suggest that the presence of the positive charge on the *p*-*N*-methyl piperazine tail is important for biochemical activity.

With the current rise of antibiotic resistance, it has become imperative that novel antibiotic strategies are developed for use in the clinic. The concept of antibiotic adjuvants (i.e., compounds which enhance the activity of established antibiotics) has gained considerable attention within the past decades,⁶ particularly after the introduction and clinical success of the β -lactamase inhibitors. Due to their roles in virulence and β -lactam resistance, a highly conserved family of bacterial kinases known as the PASTA kinases are attractive targets for the development of β -lactam adjuvants.⁹ Here, we present our findings on GW779439X, a pyrazolopyridazine protein kinase inhibitor which potentiates β -lactam activity against MRSA via inhibition of the PASTA kinase Stk1.

The intracellular kinase domain of the PASTA kinases has high structural homology to eukaryotic protein kinases, allowing for the knowledge and resources used in the eukaryotic kinase field to be easily repurposed for identification and development of bacterial PASTA kinase inhibitors. However, this also means that extra care must be taken to avoid nonspecific activity against eukaryotic kinases. Nonetheless, it is feasible to generate kinase inhibitors with relative selectivity among eukaryotic kinase targets,^{42,43} and we have previously identified an inhibitor that can discriminate between *S. aureus* Stk1 and *L. monocytogenes* PrkA, two PASTA kinases with greater similarity than that between eukaryotic kinases and PASTA kinases.²² This strongly suggests that PASTA kinase inhibitors with minimized off-target effects can be generated. Furthermore, by coadministering a relatively selective antibiotic adjuvant, there is at least the theoretical potential of focusing the β -lactam activity against the target pathogenic species and blunting the effect of the β -lactam on microbiota that do not have a PASTA kinase whose catalytic active site is highly similar to the target PASTA kinase.

To identify inhibitors of Stk1, we screened three small-molecule ATP-competitive kinase inhibitor libraries (1147 compounds total) against wild-type LAC in the presence of sublethal ceftriaxone. We chose a microbiologic approach rather than a biochemical approach as our primary screen to focus our efforts on lead compounds which could access the bacterial cytosol, a hurdle that has proven challenging in biochemical screens for inhibitors of the *M. tuberculosis* PASTA kinase PknB.³⁶ Of the 31 hits in our primary screen, we selected GW779439X as our lead compound due to its dependence on the presence of a β -lactam and Stk1, its dose responsiveness, and the accessibility of synthesized derivatives. GW779439X possesses a pyrazolo-pyridazine scaffold that is structurally unique compared to other previously identified Stk1 inhibitors.^{33–35} The sulfonamide and quinazoline scaffolds identified by Vornhagen et al. and Kant et al., respectively, were absent from the inhibitor libraries we screened. On the other hand, SB-202190, an inhibitor identified by Boudreau et al., was present in the Selleck library but failed to potentiate ceftriaxone activity against LAC, potentially due to differences in screening conditions (ceftriaxone in tryptic soy broth [TSB] reported here and oxacillin in cation-adjusted Mueller–Hinton media reported in Boudreau et al.). We validated this hypothesis by testing the compound in both conditions and found that these conditions were indeed the cause of the discrepancy

(Supplemental Figure S3). These data suggest that more potential Stk1 inhibitors could be identified by screening small molecule libraries under various growth conditions.

GW779439X potentiates the activity of a variety of β -lactam antibiotics in an Stk1-dependent manner. Interestingly, GW779439X can sensitize LAC to β -lactams to the same extent as a *stk1* mutant. This is in contrast to our previous findings with the compound GSK690693 against *L. monocytogenes* which could not reach the same levels of sensitivity as a *prkA* mutant.²² This suggests that either GW779439X has a greater efficacy against Stk1 than GSK690693 against PrkA or that the β -lactam phenotypes of Stk1 are solely dependent on its kinase activity while PrkA regulates additional effects which are independent of its enzymatic activity. The latter case would suggest that the functions of the Stk1 and PrkA signaling circuits do not completely overlap. Introduction of kinase-dead complements into their respective kinase mutants would shed light on this hypothesis.

It is intriguing that pharmacologic inhibition or genetic deletion of Stk1 potentiates different β -lactams to varying degrees. Ceftriaxone is only augmented 2-fold, while nafcillin and oxacillin (the mainstay therapies for MSSA infections) are more strikingly potentiated to below their respective clinical break points. This data suggests that while Stk1 inhibition may not be able to clinically sensitize MRSA to all β -lactams, it could allow for treatment of MRSA with a traditional MSSA therapy.

GW779439X is able to potentiate the activity of oxacillin against various *S. aureus* isolates, including both MRSA and MSSA isolates, but the potentiation is clearly strongest in PBP2A-containing strains. With the exception of USA800, the hospital-acquired MRSA isolates (COL, USA600) appear relatively recalcitrant toward GW779439X treatment, while the community-acquired MRSA isolates (USA300, USA400) are sensitized by GW779439X to below the oxacillin clinical breakpoint. USA800 is also sensitized to oxacillin though it is a hospital-acquired MRSA isolate. Further investigation is needed to elucidate the mechanism responsible for the variations in β -lactam sensitization among MRSA isolates.

GW779439X's ability to sensitize the ceftaroline-resistant MRSA strain BAA-2686 to both oxacillin and ceftaroline is quite striking and brings their MICs well below the clinical susceptibility breakpoints of 2 and 1 $\mu\text{g/mL}$, respectively. Ceftaroline's unique mechanism of action involves binding of a ceftaroline molecule into an allosteric binding site on PBP2A, leading to a conformational change which exposes the transpeptidase active site to acylation by a second ceftaroline molecule.⁴⁴ Low-level resistance to ceftaroline ($\text{MIC} > 1\text{--}8\ \mu\text{g/mL}$) can be achieved by select mutations in the allosteric binding site.^{45,46} High-level resistance ($\text{MIC} > 32\ \mu\text{g/mL}$) can be achieved directly by mutations in the PBP2A active site⁵ or indirectly by PBP2A-independent mechanisms such as mutations in PBP2, PBP4, LytD, and GdpP.^{47,48} Although the exact resistance mechanism is unknown in BAA-2686, the exquisite augmentation of ceftaroline by GW779439X might hint at underlying players in the Stk1 signaling cascade.

To begin developing a GW779439X-Stk1 SAR, we synthesized seven pyrazolopyridazine derivatives with a focus on modifications to the *p*-*N*-methyl piperazine tail and *m*-trifluoromethyl side chain. In general, we discovered that modifications to the *p*-*N*-methyl

piperazine were detrimental to biochemical activity as demonstrated by the greatly reduced activity of CAF070 and CAF075 in both our *in vitro* and microbiology assays. This could be due to the loss of a stabilizing salt bridge with Glu⁹⁷ by swapping the positively charged piperazine for an electronegative side chain (CAF070) and/or by the inability of a side chain that can reach Glu97 (CAF075) (Supplemental Figure S4). This could be further validated by reversing or removing the charge at position 97. CAF077 is unique in the fact that it simply shifts the positive charge into the *meta*-position on the aniline moiety rather than completely eliminate it. While this is ultimately a detriment to biochemical activity, some intermediate inhibition is salvaged possibly by the formation of a hydrogen bond with Thr94 (Supplemental Figure S4). However, this moderate decrease in biochemical activity is enough to potentially decrease the compound's microbiologic potency. These findings with CAF077 support the hypothesis that it is not only the presence of a positive charge, but also its optimal positioning on the side chain that is important to stabilize the compound in the active site. On the other hand, modifications to the *m*-trifluoromethyl side chain appear to be generally more forgiving; replacement with other electronegative side chains such as fluorine (CAF052) or nitrile (CAF078) retain both biochemical and microbiologic activity up par with GW779439X. Interestingly, replacement with a nonpolar methyl side chain (CAF089) greatly diminishes biochemical activity and abolishes microbiologic activity, while a complete lack of a side chain (CAF045) creates an intermediate phenotype. We speculate that an electronegative side chain at this position might add an additional stabilizing hydrogen bond with Thr94 (Supplemental Figure S4). A mutation of Thr94 to a nonpolar residue such as alanine might shed light on this hypothesis. Future work will be needed to elucidate the effects of modifications to the pyrazolopyridazine head.

Besides β -lactam sensitivity, Stk1 also regulates virulence in *S. aureus* (reviewed in ref 9). Overall, there is conflicting evidence on the effects of Stk1 interference, with certain strains and infection models showing Stk1 as a positive regulator of virulence while others reveal Stk1 to be a negative regulator of virulence. While an increase in virulence due to Stk1 inhibition may be problematic, the trade-off with increased sensitivity to antibiotics still needs to be considered. In any case, there is yet to be conclusive evidence about the effects of dysregulation of Stk1 on β -lactam therapy in an animal model of infection, a crucial experiment needed for further development of PASTA kinase inhibitors as β -lactam adjuvants.

Although it is well established that interference with PASTA kinase signaling augments β -lactam activity, our understanding of the exact signaling circuits required to maintain β -lactam resistance remains incomplete. Bacterial eSTKs have been found to phosphorylate a wide variety of candidate proteins across species such as DivIVA/Wag31,⁴⁹ Yvck/CuvA,^{50,51} MurC,⁵² VraR,⁵³ IreB,¹⁹ and GpsB.¹⁴ Of note, Stk1 has been found to play a role in regulating the activity of the class-A β -lactamase BlaZ in select *S. aureus* strains.³³ However, bacteria which lack a β -lactamase (such as *L. monocytogenes* and the *S. aureus* strains COL and Newman) are still sensitized to β -lactams when PASTA kinase signaling is disrupted,^{20,23} notably against β -lactams that are resistant to hydrolysis by BlaZ like oxacillin and nafcillin. Overall, the current literature suggests that Stk1 and other PASTA kinases most likely act as master regulators of various components of the cell wall synthesis

and division machinery, further emphasizing their attractiveness as antibiotic adjuvant targets.

CONCLUSIONS

In summary, we identified the compound GW779439X as a novel inhibitor of the *S. aureus* PASTA kinase Stk1. We have demonstrated that GW779439X potentiates the activity of β -lactam antibiotics against various MRSA and MSSA isolates, some even crossing the breakpoint from resistant to sensitive. Finally, we have determined the importance of the *p*-*N*-methyl piperazine side chain for biochemical and microbiologic activity. Future work with the pyrazolopyridazine scaffold may lead to the design of compounds with better selectivity for staphylococcal PASTA kinases as antibiotic adjuvants to restore β -lactam activity and prolong the clinical utility of newer agents such as ceftaroline.

METHODS

Bacterial Strains and Growth Conditions.

All bacterial strains used in this study are listed in Table S1. Generation of the *stk1* mutant strain was achieved as previously described.²² All *S. aureus* strains were grown in TSB medium at 37 °C shaking overnight until stationary phase. Cultures were then back-diluted to an OD₆₀₀ of 0.06 for broth growth experiments. *Escherichia coli* strains XL-1Blue and Rosetta BL21 were used for subcloning and protein expression, respectively. When needed, chloramphenicol (Sigma-Aldrich) was used at 10 μ g/mL and carbenicillin (Sigma-Aldrich) was used at 100 μ g/mL.

Library Screen.

The PKIS1, PKIS2, and Selleck libraries were obtained via the University of Wisconsin Carbone Cancer Center's Small Molecule Screening Facility. Overnight cultures were back-diluted 1:100 into fresh TSB medium containing 10 μ g/mL ceftriaxone and either library compounds (final concentration: 20 μ M in 2% DMSO) or DMSO (final concentration: 2%). Growth was measured as an optical density at 600 nm (OD₆₀₀) on 15 min intervals for 12 h in a 96-well format using an Eon microplate spectrophotometer or Synergy HT microplate spectrophotometer (BioTek Instruments, Inc., Winooski, VT) (growth conditions: 37 °C, linear shaking). Each compound was screened twice. Percent inhibition was calculated as $(1 - (OD_X/OD_{CRO})) \times 100$, where OD_X is the end point OD₆₀₀ for a culture treated with both ceftriaxone and compound X, and OD_{CRO} is the end point OD₆₀₀ for a culture treated with ceftriaxone alone. Compounds that inhibited growth three standard deviations greater than the library mean were further verified for dose responsiveness and β -lactam dependence.

MIC Determination.

S. aureus overnight cultures were back-diluted to an OD₆₀₀ of 0.06 into cation-adjusted Mueller–Hinton (CA-MH) medium containing 2-fold dilutions of the tested antibiotics in the presence or absence of 5 μ M GW779439X. The CA-MH was supplemented with 2% NaCl when determining the MICs of nafcillin, oxacillin, or ceftaroline as per recommendation by the Clinical and Laboratory Standards Institute.⁵⁴ OD₆₀₀ was measured to monitor growth of

the microdilutions for 12 h (with the exception of the *S. aureus* strain BAA-2686, which was grown for 24 h due to its relatively long lag phase). MICs were defined as the lowest concentration of antibiotic required to prevent turbidity in broth visible by eye. Each MIC experiment was performed at least three times.

Kinase Domain Protein Expression and Purification.

The *stk1* kinase domain (residues 1–348) was subcloned into the expression vector pGEX-2T as previously described.²⁰ Expression and purification of the GST-tagged Stk1 kinase domain was performed as previously described.²² Briefly, protein expression was induced by addition of 1 mM Isopropyl β -D-1-thiogalactopyranoside (IPTG) overnight at 23 °C. Cells were lysed by sonication, and the lysates were processed through GS4FF affinity resin columns. The GST tag was then cleaved by thrombin digestion, and liberated Stk1 kinase domain was further processed through a HiPrep Q16 10FF anionic exchange column and a Sephadex75 size-exclusion column to purity.

In Vitro Protein Phosphorylation.

Two micromolar kinase domain, 10 μ M MBP (Novatein Biosciences, Woburn, MA), and various concentrations of kinase inhibitors were incubated at 37 °C for 10 min, then added to a mixture of 10 mM Tris pH 7.4, 150 mM NaCl, 50 μ M MnCl₂, 50 μ M ATP, and 1 μ Ci of [γ -³²P] ATP. Reactions were incubated at 37 °C for 30 min and terminated by the addition of 6 $\times$ SDS loading buffer. Samples were run on an SDS-PAGE gel then fixed for 2 h in fixation solution (40% methanol, 5% glycerol, 10% glacial acetic acid). Fixed gels were dried for 1 h and blots visualized by autoradiography.

In Silico Modeling.

In silico modeling was performed as previously described.²² Briefly, the primary sequence of the Stk1 kinase domain (residues 1–270) was threaded onto the crystal structure of the kinase domain of PknB from *M. tuberculosis* (PDB ID 1O6Y) using the Phyre2 server's one-to-one threading.⁵⁵ Gatsieger–Huckel charges were added to Stk1 and compounds using SYBYL-X1.2.⁵⁶ Compounds were docked into a 66 $\times$ 66 $\times$ 66 unit grid encompassing the kinase's active site cleft using the docking program Autodock's Lamarckian genetic algorithm.⁵⁷ Models were visualized using PyMOL.⁵⁸

COMPOUND SYNTHESIS

Synthesis of 2-Chloro-4-((trimethylsilyl)ethynyl)-pyrimidine (Compound 2).

To a solution of Pd(dppf)₂Cl₂ (200 mg, 0.3 mmol), PPh₃ (200 mg, 0.7 mmol), and 2,4-dichloropyrimidine (1.0 g, 7.0 mmol) (Compound 1) in anhydrous THF (25 mL) was added Et₃N (33 mL) under a N₂ atmosphere. Nitrogen was bubbled into the solution for 20 min before the addition of (trimethylsilyl)acetylene (1.0 mL, 7.0 mmol) followed by CuI (100 mg, 0.7 mmol). The mixture was then heated at 70 °C for 10 min. Upon completion, the mixture was cooled to room temperature and filtered, and the solvent was removed under rotary evaporation. Purification by silica gel flash column chromatography (hexane:EtOAc 8:2) afforded 2-chloro-4-((trimethylsilyl)ethynyl)pyrimidine (Compound 2) (920 me, 4.4 mmol, 68%) as a brown solid.

Mp 49–50 °C; ^1H NMR (400 MHz, CDCl_3) δ 8.58 (d, $J = 5.0$ Hz, 1H), 7.31 (d, $J = 5.0$ Hz, 1H), 0.28 (s, 9H); ^{13}C NMR (100 MHz, CDCl_3) δ 161.6, 159.6, 152.8, 122.0, 103.7, 100.2, 0.5; LRMS m/z 211 $[\text{M} + \text{H}]^+$.

Synthesis of 3-(2-Chloropyrimidin-4-yl)pyrazolo[1,5-b]pyridazine (Compound 4).

Hydroxylamine-O-sulfonic acid (1.2 g, 10.7 mmol) was dissolved in H_2O (3 mL), and the pH was adjusted to 5–6 by the addition of a 2.5 M solution of NaHCO_3 . Pyridazine (Compound 3) (514 μL , 7.1 mmol) was added dropwise, and the solution was stirred at 70 °C for 2 h. After cooling at room temperature, the solution was neutralized to pH 8–9 by the addition of a 2.5 M solution of NaHCO_3 . Then, a solution of 2-chloro-4-[2-(trimethylsilyl)ethynyl]-pyrimidine (Compound 2) (0.5 g, 2.4 mmol) in CH_2Cl_2 (4 mL) was added, followed by a solution of KOH (0.8 g, 14.2 mmol) in H_2O (7 mL). The resulting solution turned dark red, and the stirring was continued for 18 h at room temperature. Upon completion, the mixture was diluted with CH_2Cl_2 (15 mL), and layers were separated. The organics were further extracted from the aqueous with CH_2Cl_2 (3×20 mL), and the combined organics were washed with brine (50 mL), dried over Na_2SO_4 , and filtered, and

the solvent was removed by rotary evaporation. Purification by silica gel flash column chromatography (hexane:EtOAc 7:3) afforded 3-(2-chloropyrimidin-4-yl)pyrazolo[1,5-*b*]pyridazine (Compound **4**) (0.3 g, 1.4 mmol, 58%) as a brown solid.

Mp >250 °C; ^1H NMR (400 MHz, DMSO- d_6) δ 9.03 (s, 1H), 8.90 (dd, J =9.0, 1.9 Hz, 1H), 8.73 (d, J = 5.3 Hz, 1H), 8.69 (dd, J = 4.5, 1.9 Hz, 1H), 8.06 (d, J = 5.4 Hz, 1H), 7.58 (dd, J = 9.1, 4.5 Hz, 1H); ^{13}C NMR (100 MHz, DMSO- d_6) δ 161.8, 160.3, 144.6, 140.7, 132.9, 128.9, 120.2, 115.4, 108.3; LRMS m/z 232.0 $[\text{M} + \text{H}]^+$.

General Procedure 1.

To a solution of 3-(2-chloropyrimidin-4-yl)pyrazolo[1,5-*b*]pyridazine (Compound **4**) (1.0 mmol) in *sec*-BuOH (0.05 M) was added the aniline of interest (1.3 mmol) followed by TFA (3.7 M). The resulting mixture was stirred at 100 °C for 18 h. Upon completion, the mixture was diluted with EtOAc, and a saturated solution of NaHCO_3 was added. The layers were separated, and the organics were further extracted from the aqueous with EtOAc (2 $\times$). The combined organics were washed with brine, dried over Na_2SO_4 , and filtered, and the solvent was removed by rotary evaporation. Purification by silica gel flash column chromatography (hexane:EtOAc or CH_2Cl_2 :MeOH mixtures) afforded the desired Compound **5**.

***N*-(4-(4-Methylpiperazin-1-yl)phenyl)-4-(pyrazolo-[1,5-*b*]pyridazin-3-yl)pyrimidin-2-amine (CAF045).**

Reaction of 3-(2-chloropyrimidin-4-yl)pyrazolo[1,5-*b*]pyridazine (Compound **4**) (50 mg, 0.22 mmol), 4-(4-methylpiperazin-1-yl)aniline (45 mg, 0.24 mmol) and TFA (58 μ L) in *sec*-BuOH (4 mL) according to General Procedure 1 afforded the title compound after purification by silica gel flash column chromatography (CH_2Cl_2 :MeOH 9:1) (48 mg, 0.12 mmol, 70%) as a yellow solid.

Mp 150 °C decomp; ^1H NMR (400 MHz, Methanol- d_4) δ 9.03 (dd, J = 9.1, 1.9 Hz, 1H), 8.67 (s, 1H), 8.50 (dd, J = 4.5, 2.0 Hz, 1H), 8.36 (d, J = 5.4 Hz, 1H), 7.56–7.54 (m, 2H), 7.30 (dd, J = 9.1, 4.5 Hz, 1H), 7.20 (d, J = 5.3 Hz, 1H), 7.09–7.06 (m, 2H), 3.29–3.26 (m, 4H), 2.77–2.74 (m, 4H), 2.46 (s, 3H); ^{13}C NMR (100 MHz, CDCl_3) δ 161.0, 159.9, 158.4, 148.1, 143.1, 139.4, 133.2, 131.9, 130.1, 123.2, 117.8, 116.9, 111.0, 107.4, 55.3, 49.9, 46.3; LRMS m/z 387 $[\text{M} + \text{H}]^+$; HRMS (ESI) calculated for $[\text{M} + \text{H}]^+$: 387.1967, found 387.20447

***N*-(3-Fluoro-4-(4-methylpiperazin-1-yl)phenyl)-4-(pyrazolo[1,5-*b*]pyridazin-3-yl)pyrimidin-2-amine (CAF052).**

Reaction of 3-(2-chloropyrimidin-4-yl)pyrazolo[1,5-*b*]-pyridazine (Compound **4**) (35 mg, 0.15 mmol), 3-fluoro-4-(4-methylpiperazin-1-yl)aniline (35 mg, 0.17 mmol), and TFA (41 μ L) in *sec*-BuOH (3 mL) according to General Procedure 1 afforded the title compound after purification by silica gel flash column chromatography (CH₂Cl₂:MeOH 9:1) (30 mg, 0.15 mmol, 49%) as a brown solid.

Mp 170 °C decomp; ¹H NMR (400 MHz, Methanol-*d*₄) δ 9.15 (dd, *J* = 9.2, 1.9 Hz, 1H), 8.70 (s, 1H), 8.53 (dd, *J* = 4.4, 2.0 Hz, 1H), 8.43 (d, *J* = 5.3 Hz, 1H), 7.85 (bs, 1H), 7.68 (dd, *J* = 14.8, 2.5 Hz, 1H), 7.38–7.32 (m, 2H), 7.26 (d, *J* = 5.6 Hz, 1H), 7.08 (d, *J* = 9.2 Hz, 1H), 3.18 (bs, 4H), 2.74 (bs, 4H), 2.44 (s, 3H); ¹³C NMR (100 MHz, CDCl₃) δ 160.3, 160.0, 158.3, 155.8 (d, *J* = 245.2 Hz), 143.2, 139.5, 135.8 (d, *J* = 9.2 Hz), 134.7 (d, *J* = 10.7 Hz), 133.1, 129.7, 119.2 (d, *J* = 4.8 Hz), 118.1, 116.4 (d, *J* = 3.1 Hz), 110.8, 109.6 (d, *J* = 25.3 Hz), 108.1, 55.4, 51.0 (d, *J* = 2.8 Hz), 46.3; LRMS *m/z* 405 [M + H]⁺; HRMS (ESI) calculated for [M + H]⁺: 405.1873, found 405.19572.

4-((4-(Pyrazolo[1,5-*b*]pyridazin-3-yl)pyrimidin-2-yl)-amino)-2-(trifluoromethyl)benzonitrile (CAF070).

Reaction of 3-(2-chloropyrimidin-4-yl)pyrazolo[1,5-*b*]-pyridazine (Compound **4**) (36 mg, 0.16 mmol), 4-amino-2-(trifluoromethyl)benzonitrile (32 mg, 0.17 mmol), and TFA (42 μ L) in *sec*-BuOH (3 mL) according to General Procedure 1 afforded the title compound after purification by silica gel flash column chromatography (hexane:EtOAc 3:7) (15 mg, 0.16 mmol, 25%) as a yellow solid.

Mp >250 °C; ¹H NMR (400 MHz, DMSO-*d*₆) δ 10.49 (s, 1H), 9.13 (dd, *J* = 9.1, 2.0 Hz, 1H), 8.95 (s, 1H), 8.65 (dd, *J* = 4.5, 2.0 Hz, 1H), 8.63 (d, *J* = 5.3 Hz, 1H), 8.49 (d, *J* = 2.1 Hz, 1H), 8.23 (dd, *J* = 8.7, 2.2 Hz, 1H), 8.08 (d, *J* = 8.6 Hz, 1H), 7.59 (d, *J* = 5.4 Hz, 1H), 7.51 (dd, *J* = 9.1, 4.5 Hz, 1H); ¹³C NMR (100 MHz, DMSO-*d*₆) δ 159.7, 159.0, 158.2, 145.3, 144.2, 140.1, 136.3, 132.6, 132.0 (d, *J* = 3.1 Hz), 131.5 (d, *J* = 9.8 Hz), 129.3, 128.7 (d, *J* = 11.7 Hz), 122.7 (d, *J* = 273.6 Hz), 120.8, 119.2, 116.3, 115.5 (q, *J* = 5.1 Hz), 109.7 (d, *J* = 30.7

Hz);LRMS m/z 382 $[M + H]^+$; HRMS (ESI) calculated for $[M + H]^+$: 382.0950, found 382.10433.

***N*-(4-Methoxy-3-(trifluoromethyl)phenyl)-4-(pyrazolo-[1,5-*b*]pyridazin-3-yl)pyrimidin-2-amine (CAF075).**

Reaction of 3-(2-chloropyrimidin-4-yl)pyrazolo[1,5-*b*]-pyridazine (Compound **4**) (45 mg, 0.19 mmol), 4-methoxy-3-(trifluoromethyl)aniline (41 mg, 0.21 mmol), and TFA (53 μ L) in *sec*-BuOH (4 mL) according to General Procedure 1 afforded the title compound after purification by silica gel flash column chromatography (hexane:EtOAc 3:7) (32 mg, 0.08 mmol, 43%) as a yellow solid.

Mp 220 °C decomp; ^1H NMR (400 MHz, DMSO- d_6) δ 9.65 (s, 1H), 9.06 (bd, J = 8.8 Hz, 1H), 8.90 (s, 1H), 8.62 (dd, J = 4.5, 1.9 Hz, 1H), 8.48 (d, J = 5.3 Hz, 1H), 8.09 (d, J = 2.7 Hz, 1H), (dd, J = 9.2, 2.6 Hz, 1H), 7.42 (dd, J = 9.1, 4.5 Hz, 1H), 7.38 (d, J = 5.3 Hz, 1H), 7.28 (d, J = 9.0 Hz, 1H), 3.88 (s, 3H); ^{13}C NMR (100 MHz, Methanol- d_4) δ 160.9, 160.1, 157.1, 154.0, 143.8, 139.8, 133.6, 132.2, 130.0, 126.8, 123.9 (d, J = 272.5 Hz), 121.4 (q, J = 5.7 Hz), 119.0, 113.1, 110.9, 107.9, 56.5; LRMS m/z 387 $[M + H]^+$; HRMS (ESI) calculated for $[M + H]^+$: 387.1103, found 387.11939.

***N*-(3-(4-Methylpiperazin-1-yl)phenyl)-4-(pyrazolo-[1,5-*b*]pyridazin-3-yl)pyrimidin-2-amine (CAF077).**

Reaction of 3-(2-chloropyrimidin-4-yl)pyrazolo[1,5-*b*]-pyridazine (Compound **4**) (40 mg, 0.17 mmol), 3-(4-methylpiperazin-1-yl)aniline (36 mg, 0.19 mmol), and TFA (47 μ L) in *sec*-BuOH (3 mL) according to General Procedure 1 afforded the title compound after purification by silica gel flash column chromatography (CH₂Cl₂:MeOH 9:1) (31 mg, 0.08 mmol, 46%) as a yellow solid.

Mp 139–140 °C; ¹H NMR (400 MHz, CDCl₃) δ 8.90 (dd, *J* = 9.0, 2.0 Hz, 1H), 8.49 (s, 1H), 8.40 (d, *J* = 5.3 Hz, 1H), 8.37 (dd, *J* = 4.4, 2.0 Hz, 1H), 7.33 (bs, 1H), 7.27–7.23 (m, 2H), 7.11 (dd, *J* = 9.1, 4.4 Hz, 1H), 7.07 (dd, *J* = 7.6, 1.5 Hz, 1H), 7.03 (d, *J* = 5.2 Hz, 1H), 6.69 (ddd, *J* = 8.2, 2.5, 0.9 Hz, 1H), 3.24–3.22 (m, 4H), 2.57–2.54 (m, 4H), 2.34 (s, 3H); ¹³C NMR (100 MHz, CDCl₃) δ 160.6, 159.9, 158.4, 152.0, 143.2, 140.4, 139.5, 133.2, 130.0, 129.6, 118.0, 112.4, 111.0, 110.9, 108.5, 108.0, 55.2, 48.9, 46.1; LRMS *m/z* 387 [M + H]⁺; HRMS (ESI) calculated for [M + H]⁺: 387.1967, found 387.20611.

2-(4-Methylpiperazin-1-yl)-5-((4-(pyrazolo[1,5-*b*]-pyridazin-3-yl)pyrimidin-2-yl)amino)benzonitrile (CAF078).

Reaction of 3-(2-chloropyrimidin-4-yl)pyrazolo[1,5-*b*]-pyridazine (Compound **4**) (35 mg, 0.15 mmol), 5-amino-2-(4-methylpiperazin-1-yl)benzonitrile (36 mg, 0.17 mmol), and TFA (41 μ L) in *sec*-BuOH (3 mL) according to General Procedure 1 afforded the title compound after purification by silica gel flash column chromatography (CH_2Cl_2 :MeOH 9:1) (38 mg, 0.09 mmol, 61%) as a yellow solid.

Mp 231–232 °C, ^1H NMR (400 MHz, CDCl_3) δ 8.88 (dd, J = 9.1, 2.0 Hz, 1H), 8.49 (s, 1H), 8.40–8.38 (m, 2H), 8.17 (d, J = 2.6 Hz, 1H), 7.50 (dd, J = 8.8, 2.7 Hz, 1H), 7.42 (bs, 1H), 7.24 (dd, J = 9.1, 4.4 Hz, 1H), 7.1 (d, J = 5.3 Hz, 1H), 7.04 (d, J = 8.8 Hz, 1H), 3.23–3.20 (m, 4H), 2.67–2.65 (m, 4H), 2.38 (s, 3H); ^{13}C NMR (100 MHz, CDCl_3) δ 160.2, 160.1, 158.3, 151.3, 143.3, 139.7, 134.2, 133.1, 129.4, 126.0, 125.7, 119.7, 118.5, 118.3, 110.6, 108.7, 106.8, 55.2, 51.9, 46.1; LRMS m/z 412 $[\text{M} + \text{H}]^+$; HRMS (ESI) calculated for $[\text{M} + \text{H}]^+$: 412.1920, found 412.20171.

***N*-(3-Methyl-4-(4-methylpiperazin-1-yl)phenyl)-4-(pyrazolo[1,5-*b*]pyridazin-3-yl)pyrimidin-2-amine (CAF089).**

Reaction of 3-(2-chloropyrimidin-4-yl)pyrazolo[1,5-*b*]pyridazine (Compound **4**) (33 mg, 0.14 mmol), 3-methyl-4-(4-methylpiperazin-1-yl)aniline (38 mg, 0.16 mmol), and TFA (39 μ L) in *sec*-BuOH (3 mL) according to General Procedure 1 afforded the title compound after purification by silica gel flash column chromatography (CH₂Cl₂:MeOH 9:1) (27 mg, 0.14 mmol, 47%) as a brown solid.

Mp > 250 °C; ¹H NMR (400 MHz, CDCl₃) δ 8.90 (dd, *J* = 9.1, 2.0 Hz, 1H), 8.49 (s, 1H), 8.39–8.37 (m, 2H), 7.45 (d, *J* = 2.6 Hz, 1H), 7.35 (dd, *J* = 8.5, 2.7 Hz, 1H), 7.10–7.06 (m, 3H), 7.02 (d, *J* = 5.3 Hz, 1H), 3.00–2.97 (m, 4H), 2.66 (bs, 4H), 2.42 (s, 3H), 2.34 (s, 3H); ¹³C NMR (100 MHz, CDCl₃) δ 160.7, 159.9, 158.4, 143.1, 139.5, 135.1, 133.6, 133.2, 129.9, 123.9, 119.9, 119.6, 117.9, 111.0, 107.8, 55.6, 51.5, 45.8, 18.0; LRMS *m/z* 401 [M + H]⁺; HRMS (ESI) calculated for [M + H]⁺: 401.2124, found 401.22196.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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ABBREVIATIONSUSED

PASTA	penicillin-binding-protein and serine/threonine kinase-associated
PKIS	published kinase inhibitor set
MIC	minimum inhibitory concentration
TSB	tryptic soy broth
MRSA	methicillin-resistant <i>S. aureus</i>
MSSA	methicillin-sensitive <i>S. aureus</i>
IPTG	Isopropyl β -D-1-thiogalactopyrano-side

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Figure 1.

Library screen identifies GW779439X as a compound that potentiates ceftriaxone activity against MRSA. (A) Scatter plot representing percent growth inhibition of WT LAC in the presence of a combination of a sublethal concentration (10 μg/mL) of ceftriaxone and compounds in the screen. The solid black line represents the library average (μ), and the gray line represents the three standard deviations (3σ) above the library average. The cyan and red dots represent GW779439X and other compounds from the pyrazolopyridazine family, respectively. (B) Skeletal structure of GW779439X.

Figure 2.

GW779439X directly inhibits Stk1 kinase activity *in vitro*. Autoradiography blot of purified Stk1 kinase domain and the nonspecific phosphosubstrate MBP untreated or in increasing concentrations of GW779439X. SB-747651A is an imidazopyridine aminofurazan kinase inhibitor present in the initial library screen which shows no Stk1 activity. Blot is representative of three independent trials.

Figure 3. GW779439X potentiates ceftaroline activity against a ceftaroline-resistant MRSA strain. (A) MRSA strain BAA-2686 was back-diluted into increasing concentrations of ceftaroline in the presence or absence of 5 μM GW779439X. (B) BAA-2686 was back-diluted into 5 μM GW779439X and growth was monitored for 24 h. Dose-response curves and growth curves are representative of three independent trials.

Figure 4.

The pyrazolopyridazine scaffold docks in the Stk1 active site. (A) GW779439X docked *in silico* into the Stk1 active site. GW779439X is displayed as green sticks; Stk1 residues of note are displayed as violet sticks. Stk1 backbone is displayed as violet cartoon, and Stk1 van der Waals radii are displayed as violet mesh. (B) Skeletal structure of the pyrazolopyridazine scaffold that was present in the kinase inhibitor library screen. (C) Skeletal structure of our restricted pyrazolopyridazine scaffold used for medicinal chemistry.

Figure 5.

GW779439X's *p*-*N*-methyl piperazine side chain is important for biochemical and microbiologic activity. (A) Autoradiography blot of Stk1 kinase domain and MBP untreated or in the presence of 2 μ M GW779439X (GW) or other kinase inhibitors. Blot is representative of three independent trials. (B) WT LAC was back-diluted into cation-adjusted Mueller–Hinton media with a sublethal concentration (8 μ g/mL) of oxacillin in the presence of 20 μ M GW or other kinase inhibitors. Percent inhibition for each compound was calculated relative to bacteria grown in oxacillin and DMSO. Error bars represent the standard deviation of 3 independent trials. * = statistically significant by Student's *t* test. (C) WT LAC was back-diluted into a sublethal concentration (8 μ g/mL) of oxacillin in increasing concentrations of (left) GW779439X or CAF078 or (right) CAF045, CAF052, or CAF077. Dose–response curves are representative of three independent trials.

Scheme 1. Synthesis of Pyrazolopyridazine Analogues^a

^aReagents and conditions. (a) $\text{Pd}(\text{dppf})\text{Cl}_2$ (5 mol %), PPh_3 (10 mol %), Et_3N (0.2 M), CuI (10 mol %), trimethylsilylacetylene (1.1 equiv), THF (0.3 M), 70 °C, 10 min; (b) HOSA (4.5 equiv), KHCO_3 pH 5, 70 °C, 2 h, then KHCO_3 pH 9, 2 (1.0 equiv), CH_2Cl_2 (0.6 M), KOH (aq) (6.0 equiv), rt, 18 h; (c) ArNH_2 (1.3 equiv), TFA (3.7 M), *sec*-BuOH (0.05 M), 100 °C, 18 h.

Table 1.MIC ($\mu\text{g/mL}$) of Various Antibiotics against WT LAC and *stk1* Strains $\pm 5 \mu\text{M}$ GW779439X^a

GW779439X	LAC (WT)		<i>stk1</i>	
	–	+	–	+
ceftriaxone	32 [32, 32]	16 [16, 16]	16 [16, 16]	16 [16, 32]
oxacillin	16 [16, 32]	1 [1, 4]	1 [1, 4]	1 [1, 4]
nafcillin	16 [16, 16]	2 [1, 2]	2 [1, 2]	2 [1, 2]
meropenem	0.25 [0.25, 0.5]	0.125 [0.125, 0.125]	0.125 [0.0625, 0.125]	0.125 [0.0625, 0.125]
ceftaroline	1 [0.5, 1]	0.5 [0.5, 0.5]	0.5 [0.5, 0.5]	0.5 [0.5, 0.5]
vancomycin	1 [1, 2]	1 [1, 2]	1 [1, 2]	1 [1, 2]
chloramphenicol	4 [8, 4]	4 [8, 4]	4 [8, 4]	4 [8, 4]

^aData represented as median of at least three independent trials with the range in brackets.

Table 2.MIC ($\mu\text{g/mL}$) of Oxacillin for Various *S. aureus* Isolates $\pm 5 \mu\text{M}$ GW779439X^a

GW779439X	–	+
USA400 (MW2)	16 [16, 32]	2 [1, 4]
USA600	16 [16, 16]	8 [8, 8]
USA800	4 [4, 8]	1 [0.5, 2]
COL	256 [256, 256]	64 [64, 64]
ATCC BAA-2686	128 [64, 128]	0.25 [0.25, 0.5]
Newman	0.25 [0.125, 0.5]	0.125 [0.0625, 0.125]
NCTC8325	0.125 [0.125, 0.25]	0.0625 [0.0625, 0.125]

^aData represented as median of at least three independent trials with the range in brackets.

Table 3.

Structures of Various Pyrazolopyridazine Compounds

Compound	R5	R6
GW779439X
CAF045
CAF052
CAF070
CAF075

Compound	R5	R6
CAF077		
CAF078		
CAF089		