Phenylindolylmethyldiaminopyrimidines (PIDAPs) as potent antimicrobials against Staphylococcus aureus

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Abstract

We need new antimicrobials. Phenylindolylmethyldiaminopyrimidines (PIDAPs), stop the growth of USA300 MRSA strain ATCC BAA-1717 at low micromolar concentrations. In comparison, penicillin G and vancomycin are able to stop the growth of MRSA at ~765 μM (256 μg/mL) and ~1.38 μM (2 μg/mL) respectively. Several PIDAPs were bactericidal at the MIC or two-fold higher concentrations, as was vancomycin. No activity was observed against Gram-negative pathogens. ChEMBL contains no chemicals with similar structure (Tanimoto coefficient > 0.7). This suggests PIDAPs are a novel class of chemicals with antimicrobial properties. The constitution and orientation of the pyrimidine substituents are a critical determinant of anti-MRSA activity of PIDAPs. Unfortunately, we also detected potential dose-limiting toxicity on human cell lines. Studies on the mechanism of action and how structure may be modified to enhance potency, while minimizing toxicity, are needed.

The age of antimicrobial resistance has brought forward serious clinical challenges.[1, 2] The current clinical pipeline is running dry.[3-5] Certainly, very few first-in-class antimicrobials have been approved for clinical use in the past 30 years.[6, 7] Part of the problem has been the collapse of the commercial antimicrobial R&D segment due to a variety of different reasons that affected profitability.[2, 8] To oversimplify matters, it was just not financially feasible for Big Pharma to continue investing into antimicrobial development.

This lack of novel antimicrobials has weighed heavily on healthcare as a whole. Just as the advent of antimicrobials had given healthcare professionals the ability to advance patient care unto new heights – for example, the ability to fight off infections is what enabled the development of powerful anticancer treatments, organ transplants, antiarthritic medication, etc.[9] – the advent of resistance threatens to return us to the dark ages, when it comes to preventing and curing infections. Novel antimicrobials are needed.[10]

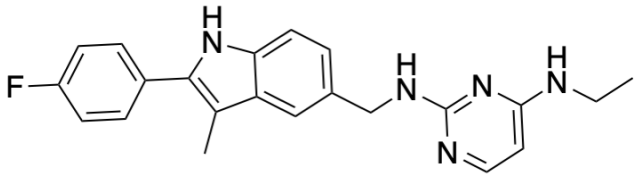

Figure 1: The prototype PIDAP (**1**). Structural variants can be observed in *Supplementary Figure S1*, and antimicrobial potency can be observed in *Table 1*, with additional details depicted in *Supplementary Table S1*.

Here, we report PIDAPs as a novel class of chemicals with powerful activity against antimicrobial-resistant strains of *Staphylococcus aureus*. The prototype PIDAP is N^4-ethyl-N^2-((2-(4-fluorophenyl)-3-methyl-1H-indol-5-yl)methyl)pyrimidine-2,4-diamine (***Figure 1***). Below, we

demonstrate our findings of antimicrobial potency and analysis of toxicity against cellular models of human tissues to demonstrate the promise and challenges associated with this chemical class, in addition to a preliminary structure-activity relationship that demonstrates the diaminopyrimidine moiety as a significant contributor to antimicrobial potency.

Growth inhibition of S. aureus strains by PIDAPS. ***Table 1*** demonstrates the growth inhibitory concentrations of PIDAPs, penicillin G (a representative β-lactam), and vancomycin against *S. aureus* USA300 MRSA strain ATCC BAA-1717. β-lactams and vancomycin are used as first-choice therapy against methicillin-sensitive S. aureus (MSSA) and MRSA respectively. Accordingly, while penicillin G was ineffective at the highest concentration tested (256 μg/mL), vancomycin was able to stop the growth of MRSA at 2 μg/mL. In comparison, some of the PIDAPS demonstrated growth inhibition at low micromolar concentrations. The best activity was demonstrated by **1** (12.5 μM or 4.7 μg/mL) and **7** (12.5 μM or 4.5 μg/mL). Negative control (broth only or broth with appropriate volume of DMSO, since the chemicals were dissolved in it) saw full bacterial growth, as expected. A similar experiment with *Escherichia coli* and *Pseudomonas aeruginosa* did not demonstrate any growth inhibition at 25 μM. However, recently reports[11] of "converting" anti-Gram-positive antimicrobials into chemicals active against Gram-negatives suggest further investigation in the same vein may be useful.

Table 1. MICs of PIDAPs against USA300 MRSA strain ATCC BAA-1717 and Mu50 VISA strain ATCC 700699.

Chemical	**MIC (μg/mL) against**	
	MRSA	*VISA*
Penicillin G	>256	32
Vancomycin	2	8
1	4.7	9.4
2	8.7	8.7
3	9.0	9.0
4	N.D.	N.D.
5	9.4	9.4
6	9.5	N.D.
7	4.5	9.0

8	8.7	N.D.
9	N.D.	N.D.
10	9.4	N.D.
11	N.D.	N.D.
12	N.D.	N.D.
13	8.7	8.7

N.D.: Not detected at highest concentration measured.

β-lactams are the primary option for treatment of infections such as infective endocarditis, caused by MSSA. However, MRSA strains can be extremely resistant to β-lactams such as penicillin G, which is clearly observed above (Table 1). Therefore, MRSA infections are treated primarily by vancomycin, where the MIC is up to 2 μg/mL – this is also reflected in Table 1. The rise of vancomycin intermediate-resitant S. aureus (VISA) strains from MRSA strains is a particularly worrisome problem because these are typically able to survive both classes of antimicrobials.[12] The treatment of VISA depends entirely on medications such as linezolid and quinupristin/dalfopristin, which are in fact, drugs of last resort. Any addition to this list will be a welcome relief. PIDAPs are potent (mostly, MIC ~10 μg/mL) compared to the prototype β-lactam, penicillin G (MIC ≥256 μg/mL) and ~5-fold less potent than vancomycin (MIC 2 μg/mL). Interestingly, several of the PIDAPs (**6**, **8** and **10**) that were able to inhibit MRSA growth were not active against VISA at the highest concentration tested (~5-fold higher). Others, such as **1**, **7**, and **10** are slightly less potent against VISA, as they are against MRSA. One major difference between MRSA and VISA strains is the increased cell wall thickness.[13, 14] We therefore hypothesize this could potentially explain why some PIDAPs were less potent against VISA, although we also concede this could simply be an artefact of the MIC testing procedure. Further work is needed to confirm such matters, even though our data was fully reproducible to within the expected 2-fold dilution error.

Minimum bactericidal concentration (MBC) determination. We also identified the concentrations at which vancomycin and PIDAPs were bactericidal against MRSA. Vancomycin

was bactericidal at its MIC, as were most of the PIDAPs (except 7, which gave a 2-fold change across replicates). Penicillin G was not studied because it did not show growth inhibition even at the highest concentration we used in MIC assays (256 μg/mL). This bactericidal activity is, of course, highly promising because vancomycin can be such an effective treatment for *S. aureus* infections. We therefore find that the mechanism of action for PIDAPs must be investigated in the future to ascertain how they function.

Kill curves. We compared the rate at which PIDAP **1** kills MRSA to penicillin G and vancomycin. We used a much denser culture of MRSA ($10^{7\text{-}8}$ CFU/mL) to achieve this than we would to measure MICs, in order to facilitate the use of a plate reader. The final concentration of **1** in the assay was 18.75 μg/mL (50 μM), while penicillin G and vancomycin were at 512 μg/mL and 2 μg/mL respectively. **1** was rapidly able to reduce optical density of MRSA in the culture with minimal variance (***Figure 2***). While vancomycin was close in its ability to reduce bacterial density, MRSA grew even at this high concentration of penicillin G.

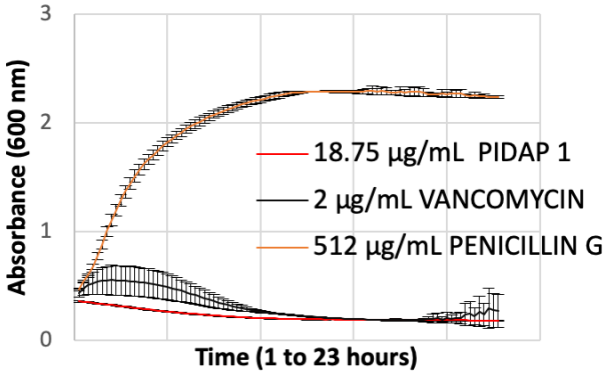

Figure 2: Growth/kill curves for PIDAP 1, penicillin G, and vancomycin against MRSA. Growth (or lack thereof) was measured using a shaking-incubating plate reader set at 37 °C. MRSA concentration was set at 10^{7}-10^{8} CFU/mL for this experiment to allow detectable absorbance at 600 nm, which is 100 to 1000-fold higher than in antibiotic susceptibility testing. While penicillin G failed to stop the growth of MRSA at 512 μg/mL, 2 μg/mL vancomycin and 18.75 μg/mL PIDAP 1 stopped the growth of the pathogen. Error bars (±SD) are shown for 2 runs.

Effect of structure on activity. ***Figure 3*** and *Supplementary Figure S1* summarize our findings. We found that the pyrimidine group could only tolerate a few alterations. The presence of an amine at the 4-position and the aromatic nitrogen at the 3-position were critical to activity. Removal of either eliminated antimicrobial potency at the highest concentration tested (50 μM). 4-ethylamino substituent was optimal, but methyl and isopropyl substituents were also tolerated. We have not yet assessed the effect of bulkier substituents at this location. Interestingly, while a 5-fluoro substitution was tolerated (MIC 25 μM), a methyl group did not show inhibition. Cumulatively, this suggests that the pyrimidine sits in a tight pocket anchored by hydrogen bonds formed between an unknown target and the nitrogen at the 3-position and the 4-amine. On the other hand, we have only sparsely investigated alterations to the rest of the chemical structure. *para*- and *ortho*-fluorophenyl substitution to the indole function equally well. It is possible that further structural alterations to this end of the molecule will enhance our knowledge of the pharmacophore, and potentially even allow us to overcome toxicity to human tissues in the future.

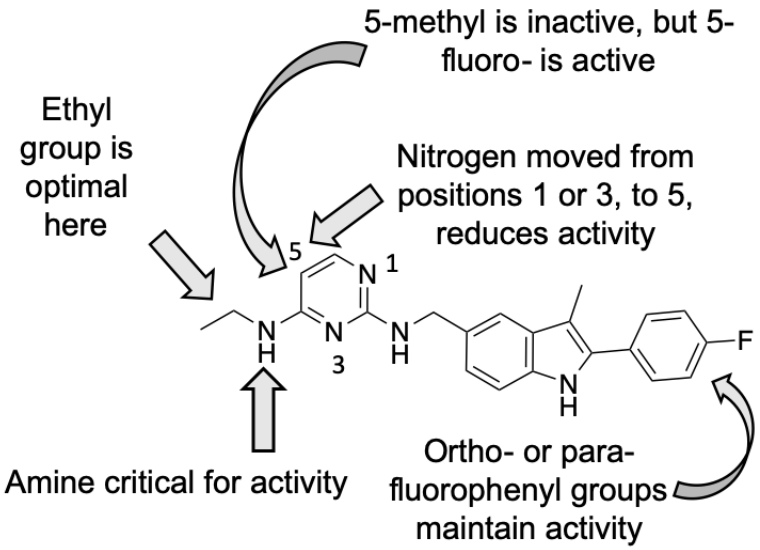

Figure 3: The effect of structure on antimicrobial activity of PIDAPS.

PIDAPs show cytotoxicity in human cell lines. In the development of novel antibiotics, it is crucial to determine any impact on mammalian cells. Therefore, in order to investigate any impact of PIDAPs on human cell lines we treated normal human cells (immortalized Schwann cells, iHSF-1λ) and human cancer cells (malignant peripheral nerve sheath tumor, S462-TY) with increasing concentrations of PIDAP **1**. Using MTT viability assays, we found that PIDAP **1** showed cytotoxicity of both cell lines tested (***Figure 4***). Both normal and cancer cell lines showed similar loss of viability the presence of PIDAP **1** in a dose dependent manner.

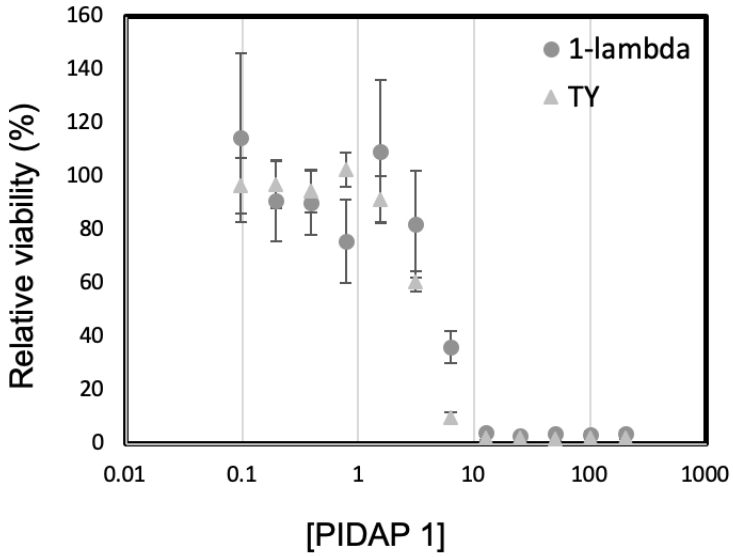

Figure 4: PIDAP 1 shows cytotoxicity in immortalized Schwann cells, iHSF-1λ) and human cancer cells at ~10 μM.

PIDAPs occupy unique chemical space. There are no reports of similar chemicals (Tanimoto coefficient[15] ≥ 0.5), antimicrobial or otherwise, in ChEMBL,[16-18] a comprehensive literature-derived database annotating biological activity to chemicals. An additional search in SciFinder[19] also failed to yield reports of biological activity. This strongly suggests PIDAPs have not been investigated well so far. We even attempted to identify indolylmethyldiaminopyrimidines analogs in ChEMBL, but found none at the same similarity level. Further, we evaluated annotated activities of chemicals containing the diaminopyrimidine group because our investigations demonstrate it is responsible for the antimicrobial properties of PIDAPs. A substructure search found over 3,000

chemicals with biological activities marked. Two of these were dapivirine and rilpivirine (non-nucleoside reverse transcriptase inhibitors) – the only known anti-infectives within this search. We also found pazopanib, a medication used against advanced stage kidney cancer that inhibits multiple receptor tyrosine kinases, among these results. Both, reverse transcriptase and kinases bind nitrogenous bases. We therefore hypothesize that PIDAPs may target MRSA and VISA kinases. This is mere speculation based on very thin grounds, of course, but we will attempt to identify the target of PDAPs in future studies.

Conclusions

PIDAPS possess antimicrobial activity against common MRSA and VISA strains, but are ineffective against Gram-negative pathogens. They are much more potent than a prototype β-lactam against MRSA and VISA, and comparable to vancomycin. The potency is even more lucrative, seeing as all the PIDAPs we have tested are closer to lead-like[20] than drug-like.[21, 22] This means there is significant room for structure-function work. However, PIDAPs are also cytotoxic against a model of human tissues and a human cancer cell line (*vide supra*). These cell lines are known to be hardy, meaning clinical development of PIDAPs will become possible only after identifying ways to eliminate toxicity while maintaining or enhancing antimicrobial potency. Overall, PIDAPs represent a novel series of chemicals that must be investigated as possible antimicrobials.

Methods

Sources of chemicals and reagents. PIDAPs were purchased from ChemBridge. All other chemicals and reagents were purchased from Sigma Aldrich and/or Fisher Scientific.

Table 2. Strains and probes used in this study.

Strain/probe	Description	Source
Bacterial species/strains (catalog #)		
USA300 MRSA (BA-1717)	Methicillin-resistant, vancomycin-susceptible	ATCC
Mu50 Rosenbach VISA (700699)	Methicillin-resistant, vancomycin-intermediate resistant	ATCC
Castellani & Chalmers *E. coli* (25922)	β-lactam susceptible	ATCC
P. aeruginosa (155250A)	MicroKwik culture	Carolina Biological Supply Company

Determination of MIC. The minimum concentration of a chemical necessary to stop the growth of bacteria (MRSA, VISA, E. coli, and P. aeruginosa) was determined by treating ~$5e^5$ CFU/mL of the pathogen with varying concentrations (a series of 2-fold dilutions) of the chemical in a 96-well plate. The growth medium used was cation adjusted Mueller-Hinton Broth. The maximum and minimum concentrations of various chemicals tested in these assays are noted: vancomycin (32 μg/mL, 0.25 μg/mL), penicillin G (256 μg/mL, 2 μg/mL), PIDAPs (25 μM, ~0.195 μM). The plates were stored for 15 hours at 37 °C in an incubator, after which, growth of bacteria was recorded at 600 nm using a plate reader.

MBC determination. The wells from MIC plates where no growth was observed, were diluted 10^3-fold in nutrient broth. This neutralizes the effects of antimicrobials or PIDAPs, which allows any surviving colony forming units to proliferate. The concentration of a PIDAP or antimicrobial at which growth was not observed even after such dilution, was marked as the MBC.

Growth/Kill curves. Stock solutions of chemicals were diluted to desired concentration in MHB II. It was then inoculated using an overnight culture of bacteria to achieve the desired concentration of ~$5e^7$ CFU/mL, and placed into a shaking incubator at 37 °C. Growth was monitored using absorbance at 600 nm. The mean and SD of duplicate experiments were recorded.

Cell Culture. Normal immortalized Schwann cells (iHSF-1λ) were kindly gifted by Margaret Wallace (University of Florida, Gainesville, FL). S462TY cells were previously described in reference[23]. All cells were used within 2 months of cryoressurection for these studies. All cell lines were maintained in DMEM supplemented with 10% fetal bovine serum, 250 U/ml penicillin, and 250 µg/ml streptomycin at 5% CO_2/37 ºC.

MTT Viability Assay. Cells were grown to log phase, trypsinized, washed in PBS, and plated in 96-well plates. iHSF-1λ and S462TY cells were seeded at 6,000 and 4,000 cells per well, respectively, in 100 µl medium. The following day, cells were treated with 2x PIDAP **1** (0-200 µM) or vehicle in normal growth medium (100 µl). Cells were incubated at 5% CO_2 at 37 ºC for 72 hours. MTT reagent (5.0 mg/ml in PBS) was added to achieve a final concentration of 0.5 mg/ml MTT. Cells were incubated for 45-75 minutes. Media was removed and cells lysed in 200 µl dimethyl sulfoxide. Wells were mixed and absorbance read at 560 nm and corrected for background at 650 nm using a plate reader.

Search for similar structures and biological activities. We used the ChEMBL[16-18] database website[24] and SciFinder[19] to identify chemicals with similar structures, and the literature-derived biological activities associated with them. Substructure searches and similarity searches are made possible by the tools built into the database. Chemical structures were drawn into the search tool and appropriate settings (percent similarity of 0.7 or 0.5 for similarity searches/none for the substructure search) were used. Results were checked for annotations of biological activity.

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SUPPLEMENTARY INFORMATION

Phenylindolylmethyldiaminopyrimidines (PIDAPs) as potent antimicrobials against Staphylococcus aureus

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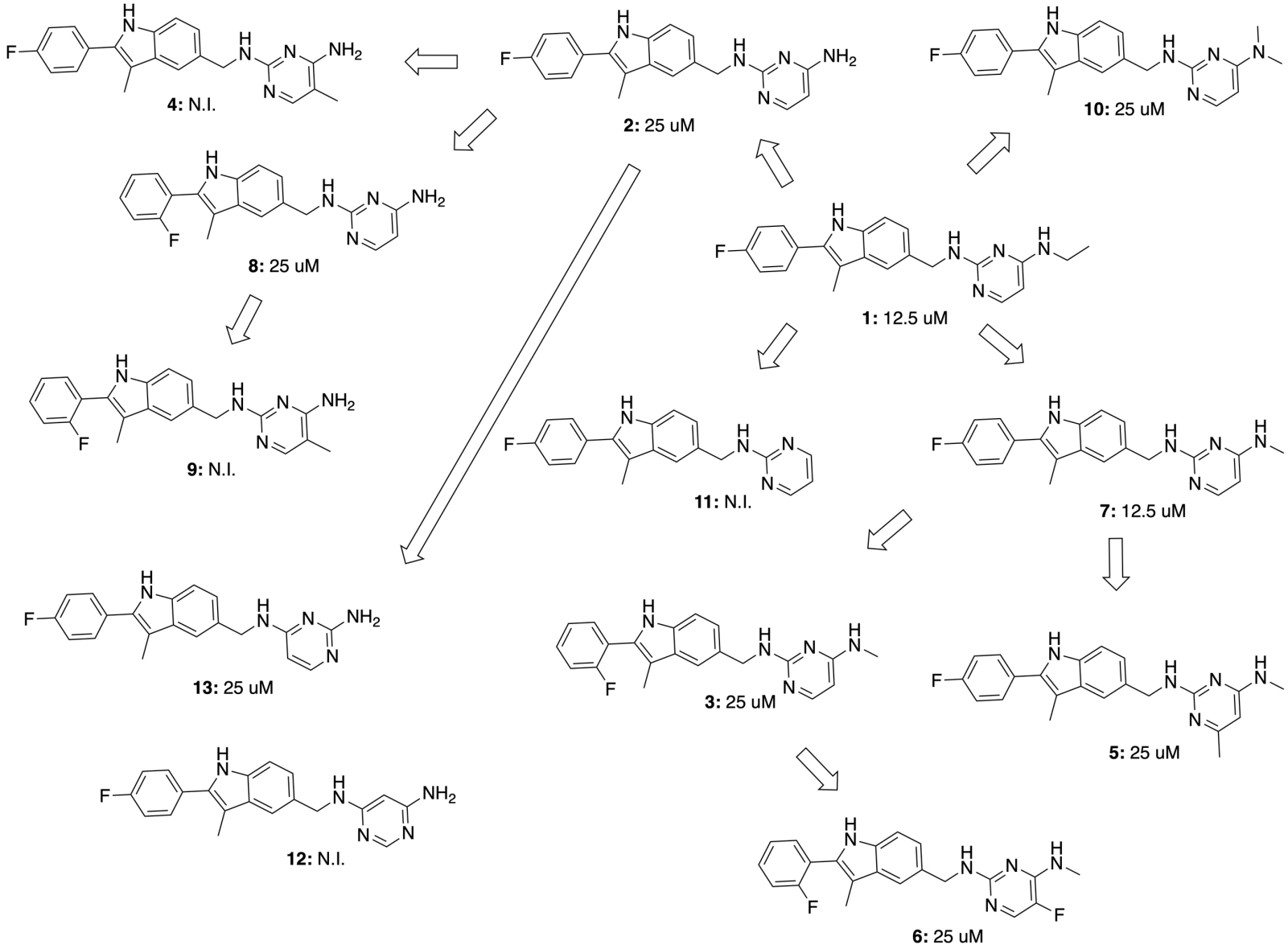
Supplementary Figure S1. Structural variations studied in this manuscript, arranged to demonstrate the structure-activity relationship against MRSA.



Supplementary Table S1. Chemical structures, MICs, MBCs, and ChemBridge catalog IDs of PIDAPs and antibiotics.

ID	ChemBridge catalog ID	Mol Wt (g/mol)	MRSA		VISA	
			MIC (μM)	MIC (μg/mL)	MIC (μM)	MIC (μg/mL)
1	38535120	375	12.5	4.6875	25	9.375
2	83912116	347	25	8.675	25	8.675
3	15789603	361	25	9.025	25	9.025
4	21735370	361				
5	41317895	375	25	9.375	25	9.375
6	75716333	379	25	9.475		
7	21681180	361	12.5	4.5125	25	9.025
8	77707091	347	25	8.675		
9	80200870	361				
10	18938981	375	25	9.375		
11	99754289	332				
12	84058648	347				
13	21611296	347	25	8.675	25	8.675
Pencillin G		334	>766.5	>256	95.8	32
Vancomycin		1449	1.4	2	5.5	8