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A scaffold hopping strategy to generate new aryl-2-amino pyrimidine MRSA biofilm inhibitors†

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Infections that stem from bacterial biofilms are difficult to eradicate. Within a biofilm state, bacteria are upwards of 1000-fold more resistant to conventional antibiotics, necessitating the development of alternative approaches to treat biofilm-based infections. One such approach is the development of small molecule adjuvants that can inhibit/disrupt bacterial biofilms. When such molecules are paired with conventional antibiotics, these dual treatments present a combination approach to eradicate biofilm-based infections. Previously, we have demonstrated that small molecules containing either a 2-amino pyrimidine (2-AP) or a 2-aminoimidazole (2-AI) heterocycle are potent anti-biofilm agents. Herein, we now report a scaffold hopping strategy to generate new aryl 2-AP analogs that inhibit biofilm formation by methicillin-resistant *Staphylococcus aureus* (MRSA). These molecules also suppress colistin resistance in colistin resistant *Klebsiella pneumoniae*, lowering the minimum inhibitory concentration (MIC) by 32-fold.

Introduction

Enterococcus faecium, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, and the *Enterobacter* species are opportunistic bacteria dubbed the ESKAPE pathogens and are notorious for their impact on human health.[1] Multi-drug resistant isolates of these pathogens pose some of the most serious health threats, as a result of a diminishing number of treatment options.[1] Of the ESKAPE pathogens, methicillin resistant *S. aureus* (MRSA) remains one of the highest in terms of infections and mortality, and is responsible for approximately 80 000 severe infections, which result in over 10 000 deaths annually in the United States alone.[2]

Outside of acquired resistance mechanisms, one way in which many bacteria evade the action of antibiotics is by adopting a biofilm phenotype. Biofilms are surface-associated communities of microorganisms surrounded by an extracellular matrix made up of biomolecules called the extracellular polymeric substance (EPS). As a result of several factors, bacterial cells within a biofilm exhibit up to 1000-fold increased tolerance to antibiotics than their free-floating (planktonic) counterparts. Such factors include protection imparted by the biofilm matrix itself, higher cell density, and varying metabolic rates of cells within the biofilm.[3–6] Biofilms are typically present in chronic, difficult/impossible to treat infections including lung infections of cystic fibrosis (CF) patients, infections of indwelling medical devices, as well as chronic and diabetic wounds. For this reason, inhibiting biofilm formation and/or dispersing preformed biofilms is one approach to aid in combatting these recalcitrant infections.

Although the development of small molecules with anti-biofilm activity is an attractive approach to fighting chronic bacterial infections, there are still relatively few chemical scaffolds that have been developed that are effective at modulating the bacterial biofilm life cycle.[7] Our group, among others, has disclosed a handful of structures with such activities.[8] Many of our earlier reported scaffolds focused on functionalized 2-aminoimidazoles (2-AIs), seen in compound **1** (Fig. 1), to harness structural similarities to marine alkaloid natural products that display anti-biofilm activity against marine biofilms (commonly referred to as

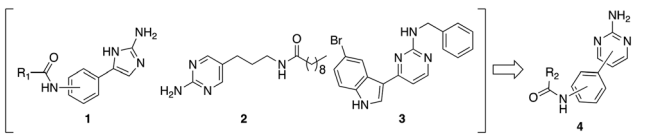

Fig. 1 Previous biofilm life cycle modulating molecules and new scaffold design.

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microfouling). Analogues of **1** were shown to inhibit the formation of *Escherichia coli* and *P. aeruginosa* biofilms.[9] Later studies demonstrated that derivatized 2-aminopyrimidines (2-AP), as seen in compounds **2** and **3** (Fig. 1) inhibited the formation of MRSA biofilms[10,11] In an initial study, compound **2** was the most active derivative, displaying an IC_{50} of 72 μM (where the IC_{50} is defined as the concentration at which a compound inhibits 50% of biofilm formation compared to untreated bacteria).[10] Recently, we posited that the meridianins, a family of natural products that contain a 2-AP subunit, possessed anti-biofilm activity. To test this hypothesis, we synthesized a family of meridianin D derivatives and discovered that compound **3** (Fig. 1)[11] inhibited the formation of MRSA biofilms and surprisingly suppressed colistin resistance in colistin resistant Gram-negative bacteria. Given the activity of these 2-AP analogs, we hypothesized that a scaffold hopping approach centered around replacing the 2-AI of **1** with a 2-AP would deliver compounds with anti-biofilm activity and/or the ability to suppress colistin resistance. By replacing the 2-AI with the 2-AP we remove a hydrogen bond donating group and increase the size of the ring. Additionally, we change the placement and identity of the aromatic rings attached to the 2-AP which proved effective on compound **3**. In order to probe the SAR of this proposed class of aryl 2-AP analogs, we synthesized a library of compounds utilizing six distinct orientations: *ortho*, *meta*, and *para* substituted phenyl core with either C-4 or C-5 attachment to the pyrimidine head group (**4**, Fig. 1).

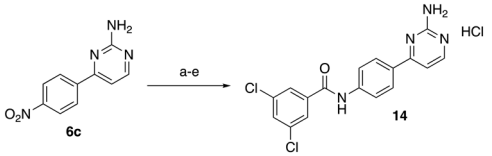
Results and discussion

Synthesis

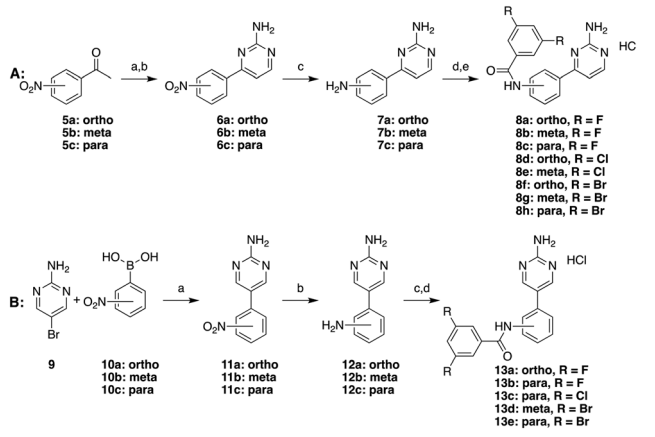
The synthetic approach to access C-4 linked 2-AP derivatives is outlined in Scheme 1A. Each acetophenone (**5a–c**) was reacted with DMF/dimethylformamide-dimethylacetal (DMF-DMA) to generate their corresponding vinylogous amide, which underwent cyclization with guanidine hydrochloride to afford the C-4 substituted 2-AP nitro intermediates **6a–c**. Reduction of the nitro group followed by coupling with the appropriate derivatized benzoyl chloride in the presence of tri-basic potassium phosphate allowed access to the aryl 2-AP analogs. After purification, each 2-AP analog (**8a–h**) was then converted to the corresponding HCl salt for biological testing. C-5 linked derivatives were synthesized using the route depicted in Scheme 1B. Each boronic acid (**10a–c**) was cross-coupled with 2-amino-5-bromopyrimidine to afford 2-AP derivatives **11a–c**. Reduction, coupling, and conversion to the HCl salt as above generated target analogs **13a–e**.

While this synthetic route was used for the majority of analogues, initially we attempted to mask the reactivity of the exocyclic pyrimidine amine using Boc protection. Although this ultimately proved unnecessary, five analogues (compounds **14**, and **15a–d**) were synthesized *via* this lengthier route that involved protection and deprotection steps. To synthesize the C-4 linked 2-AP with a 3,5-dichlorobenzoyl tail (**14**, Scheme 2), intermediate **6c** was di-Boc protected using Boc-anhydride and DMAP. Following Boc-protection, reduction, acylation, and purification proceeded smoothly. The intermediate 2-AP was then deprotected using TFA in DCM and converted to its corresponding HCl salt for testing.

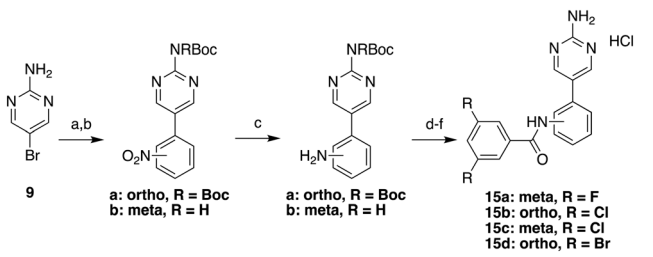
The four remaining compounds were accessed using the synthetic approach summarized in Scheme 3. 2-Amino-5-bromopyrimidine (**9**) was Boc protected using Boc anhydride and pyridine. Following Boc protection, a Suzuki reaction was carried out as before with either 2- or 3-nitroboronic



Scheme 2 Reagents and condition for synthesis of compound **14**: (a) Boc-anhydride, DMAP, DCM, 18 h; (b) H_2, Pd/carbon, rt, 16 h; (c) 3,5-dichlorobenzoyl chloride, K_3PO_4, dry THF, 0 °C to rt, 10 h; (d) TFA, DCM (e)HCl/MeOH.



Scheme 1 Reagents and conditions for general synthesis: A: (a) DMF-DMA, 110 °C 4 h; (b) guanidine hydrochloride, 2-methoxyethanol, reflux, 16 h; (c) H_2, Pd/carbon, rt, 16 h; (d) ArCOCl, K_3PO_4, dry THF, 0 °C to rt, 10 h; (e) HCl/MeOH; B: (a) $PdCl_2(PPh_3)_2$, Na_2CO_3, dry THF, reflux 20 h; (b) H_2, Pd/carbon, rt, 16 h; (c) ArCOCl, K_3PO_4, dry THF, 0 °C to rt, 10 h; (d) HCl/MeOH.



Scheme 3 Reagents and condition for synthesis of compounds **15a–d**: (a) Boc-anhydride, pyridine 18 h (b) boronic acid, $PdCl_2(PPh_3)_2$, Na_2CO_3, dry THF, reflux 20 h; (c) H_2, Pd/carbon, rt, 16 h; (d) corresponding benzoyl chloride, K_3PO_4, dry THF, 0 °C to rt, 10 h; (e) TFA, DCM (f) HCl/MeOH.

Table 1 IC_{50}'s of aryl 2-APs against MRSA 43300 biofilms

Compound	**8a**	**8b**	**8c**	**8d**	**8e**	**8f**
IC_{50} (μM)	>200	>200	>200	>50	26.4 ± 4.9	>200
Compound	**8g**	**8h**	**13a**	**13b**	**13c**	**13d**
IC_{50} (μM)	17.4 ± 6.4	>50	>200	>200	>200	43 ± 8.3
Compound	**13e**	**14**	**15a**	**15b**	**15c**	**15d**
IC_{50} (μM)	>100	41 ± 12	>200	>100	>200	69 ± 16

acid. With 2-nitro boronic acid, we noted that both Boc groups remained intact after cross-coupling, while reaction with 3-nitroboronic acid resulted in cleavage of one of the Boc groups. Following reduction of the nitro-groups to their corresponding anilines, acylation was carried out as before. Finally, each 2-AP analog was deprotected using TFA in DCM, that, following salt exchange, delivered compounds **15a–d** (Scheme 3).

Biological evaluation

All compounds were first evaluated for their ability to inhibit MRSA biofilms. Initially, each compound was tested at 200 μM against MRSA (strain ATCC 43300) and inhibition was measured using a crystal violet assay as previously reported.[12] Compounds that exhibited greater than 50% inhibition at 200 μM were then subjected to a dose response assay to determine their IC_{50} values (Table 1).

From the 18-compound pilot library, only five analogues had IC_{50}'s less than 100 μM (Table 1). Two of these analogues contained the 3,5-dichloro tail while the other three compounds possessed the 3,5-dibromo tail. The highest activity was observed when the 2-AP was substituted at C-4 (*versus* the C-5) and the amide linker was positioned *meta* to the 2-AP (**8e** and **8g**). These two compounds displayed IC_{50}'s of 26.4 ± 4.9 μM (**8e**) and 17.4 ± 6.4 μM (**8g**). Compounds **14**, **13d** and **15d** all displayed moderate activity. Analog **14** (IC_{50} = 41 ± 12 μM) possessed a 3,5-dichloro tail placed at the para position of the phenyl core and substituted at C-4 of the 2-AP. Compound **13d** (IC_{50} = 43 ± 8.3 μM) had a 3,5-dibromo tail placed at the meta position of the phenyl core and substituted at C-5 of the 2-AP. Finally, **15d** (IC_{50} = 69 ± 16 μM) had the 3,5-dibromo tail placed at the ortho position of the phenyl core and substituted at C-5 of the 2-AP.

We next tested the two most active compounds, **8e** and **8g**, for their anti-biofilm activity against additional MRSA strains, as well as a methicillin susceptible *S. aureus* strain. Both compounds had comparable IC_{50}'s across each of the four strains tested (Table 2) demonstrating that these compounds are active against multiple MRSA strains. Following this, we sought to determine whether these compounds were specifically inhibiting biofilm formation, and not simply inhibiting planktonic growth under the conditions of the biofilm assay. To that end, we constructed time kill curves using strain 43 300 as our test bacterium and compared growth in the absence and presence of compounds **8e** and **8g** at their IC_{50} concentration (Fig. S1†). Growth curves in the absence and presence of either compound were similar, establishing that these compounds are not toxic to planktonic bacteria and could potentially demonstrate a lower frequency of resistance.

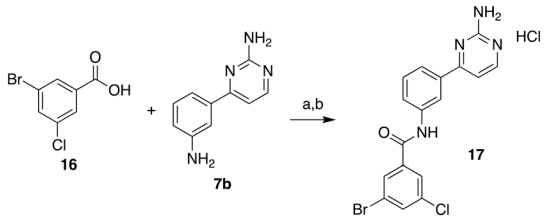
Next, we screened our compounds for their ability to disperse preformed biofilms. None of the 18 compounds demonstrated any dispersion activity against MRSA ATCC 43300 at a concentration of 100 μM.

Due to the activity of the *meta* 3,5-dichloro (**8e**) and the *meta* 3,5-dibromo (**8g**) analogues, we elected to construct and screen a hybrid analogue containing one chloro and one bromo substituent. To access this derivative, we acylated compound **7b** with 3-bromo-5-chlorobenzoic acid (**16**) under standard EDC coupling conditions. Purification and conversion to the corresponding HCl salt as previously described yielded compound **17** (Scheme 4). With this compound in hand, we first assessed the MIC of the compound and determined it to be >200 μM. We then evaluated its biofilm inhibition properties and determined that its IC_{50} was 27.6 ± 9.6 μM, which is within the error of both **8e** and **8g**.

Finally, having previously shown that analogs of the 2-aminopyrimidine-containing marine sponge natural product meridianin D potentiated colistin in Gram-negative bacteria, we probed whether these compounds also potentiated colistin. To that end, we first determined the minimum inhibitory concentration (MIC) of each compound against *A. baumannii* (AB 4106) and *K. pneumoniae* (KP B9), both of which have chromosomally encoded colistin resistance. All 18 compounds registered an MIC of ≥200 μM. We then screened each compound at 60 μM in combination

Table 2 IC_{50}'s of lead aryl 2-AP analogues against panel of *S. aureus* strains biofilms

Compound	MRSA 43300 (μM)	MRSA BAA-44 (μM)	MRSA 33591 (μM)	*S. aureus* 6538 (μM)
8e	26.4 ± 4.9	29.5 ± 2.1	26.6 ± 4.1	24.9 ± 4.2
8g	17.4 ± 6.4	14.1 ± 2.2	20.2 ± 3.0	15.6 ± 6.4



Scheme 4 Reagents and condition for synthesis of compound **17**: (a) EDC, DMAP, DCM, 18 h (b) HCl/MeOH.

with colistin to determine the effect on colistin activity. The MIC of colistin was 1024 and 512 μg mL^{-1} against AB 4106 and KP B9, respectively, as previously reported.[11,13]

None of the compounds lowered the MIC of colistin against AB 4106 below 64 μg mL^{-1} at 60 μM. However, one compound from this pilot library, compound **8g**, did lower the MIC of colistin from 512 to 16 μg mL^{-1} against KP B9, a 32-fold reduction. Interestingly, compound **8g** was also the most active MRSA biofilm inhibitor out of the 18-compound library. With antibiotic-adjuvants, we look for adjuvants that can lower the MIC of an antibiotic to its breakpoint against a bacterial strain. The colistin breakpoint in AB 4106 and KP B9 is 2 μg mL^{-1}; therefore, further diversification of this scaffold may allow for a new class of molecules that can potentiate colistin activity.

To determine if **8g** exhibits synergy with colistin, we performed checkerboard assays with compound **8g** and colistin in KP B9, and observed a fractional inhibitory concentration index (FICI) of ≤0.09 (Table S3†). We also performed the checkerboard assay in two additional strains of *K. pneumoniae*; A5, an additional highly colistin resistant clinical isolate, and F2210219$^{mcr\text{-}1}$, an engineered strain harboring a plasmid containing the *mcr-1* gene.[14] In both strains, compound **8g** exhibited synergy with colistin, returning FICI values of ≤0.31 and ≤0.19 for A5 and F2210219$^{mcr\text{-}1}$ respectively (Table S3†).

Resistance to colistin typically involves modification of the lipid A anchor of the lipopolysaccharide (LPS) of Gram-negative bacteria. To begin probing the mechanism of action for this decrease in colistin resistance we analyzed lipid A extracted from KP B9 grown in the absence and presence of **8g** *via* mass spectrometry. No change in the lipid A substitution pattern was noted between the two samples, indicating that these compounds potentiate colistin through a mechanism not dependent upon reversing lipid A modification. Further investigation into the mechanism of action is ongoing.

Finally, we tested the hemolytic activity of compounds **8g** and **8e** by performing a hemolysis assay using defibrinated sheep's blood challenged with either compound. Triton-X (1%) used as the 100% lysis marker, and phosphate buffered saline as the 0% lysis marker. Compounds **8e** and **8g** only lysed 5% and 3.4% of cells respectively when dosed at 200 μM, a concentration higher than the IC_{50} of either compound suggesting that there would be little to no lysis of eukaryotic cells when dosed at a lower concentration.

Conclusions

In conclusion, we have employed a scaffold hopping strategy to develop compounds with anti-biofilm activity against MRSA. While the lead compound **8g** shows comparable inhibitory activity with previously reported compounds, none of the disclosed compounds were able to disperse preformed biofilms. The lead compound **8g** also suppressed resistance to the polymyxin antibiotic colistin in *K. pneumoniae*, encouraging future structural derivatizations that could increase this activity. We determined that the specific connectivity between the 2-AP head, the phenyl core, and the aryl tail has a significant effect on activity, and studies to diversify this scaffold further are underway.

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Conflicts of interest

Dr. C. Melander is a co-founder of Agile Sciences, a biotechnology company seeking to commercialize antibiotic adjuvants.

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