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Potentiation of *Francisella* resistance to conventional antibiotics through small molecule adjuvants†

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A screen of 20 compounds identified small molecule adjuvants capable of potentiating antibiotic activity against *Francisella philomiragia*. Analogue synthesis of an initial hit compound led to the discovery of a potentially new class of small molecule adjuvants containing an indole core. The lead compound was able to lower the MIC of colistin by 32-fold against intrinsically resistant *F. philomiragia*.

Introduction

Francisella are small, facultative Gram-negative bacteria that are responsible for zoonotic disease in which humans are infected, usually from bites or contact with infected blood.[1] The most common vectors of disease are ticks and wild rabbits.[2] *Francisella* can also be a highly infectious aerosol and has the potential to be weaponized.[3] Encompassed under the *Francisella* genus are two species of consequence: *Francisella philomiragia* and *Francisella tularensis*. Very resilient, the bacteria are capable of surviving multiple weeks in the environment and have been found in water sources around the world.[4] Although, not as virulent and somewhat rare in humans,[5] *F. philomiragia* is a potential model organism to study *Francisella*. Human cases of *F. philomiragia* are often associated with individuals with weakened immune systems or who have suffered from a near drowning experience.[6] *F. philomiragia* is also the causative agent of the disease francisellosis, most common among various species of fish.[7] Francisellosis outbreaks have been responsible for mortality rates of up to 95% within farmed fish.[8]

Our laboratory is interested in using small molecule adjuvants to potentiate antibiotic activity. We have shown that 2-aminoimidazoles (2-AIs) are capable of potentiating antibiotic activity against a wide variety of bacteria.[9–13] We completed a screen of randomly chosen molecules from our in house library and identified four compounds, all of which, did not contain a 2-AI or 2-ABI core, but another nitrogen containing heterocycle (indole, indoline, or oxazoline) (Fig. 1) that were capable of potentiating antibiotic response in *F. philomiragia*. A structure activity relationship (SAR) study of compound 4 was carried out to discover potentially more potent leads and to better understand the structural requirements for activity. Herein, we report the discovery of the first example of a potentially new class of lead compounds containing an indole core capable of breaking *F. philomiragia* resistance to the antibiotic colistin.

Results and discussion

Adjuvant screen for MIC suppression of antibiotics against *F. philomiragia*

An initial pilot screen of 20 compounds from our in-house small molecule library[14–18] was conducted by first determining

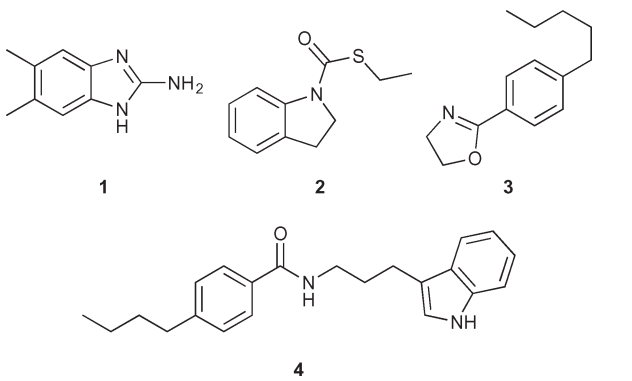

Fig. 1 Lead structures identified from initial screening.

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the intrinsic antibiotic activity of each molecule by establishing its minimum inhibitory concentration (MIC) against *F. philomiragia* (Fig. S1, ESI†). Following our lab's previously reported screening protocol,[9] the MIC of candidate conventional antibiotics were then determined in the absence or presence of each compound at 25% of the compound's MIC. Previous studies from our group has established that molecules from our internal library had little (if any) effect on bacterial growth at 25% MIC, allowing us to study non-microbicidal repotentiation of the conventional antibiotics under study. Streptomycin, an aminoglycoside, was chosen as the initial antibiotic to screen for potential adjuvants as it is considered to be the drug of choice for tularemia treatment.[19] Initial testing with streptomycin revealed of the 20 compounds screened only compound **1** showed activity, a reduction in MIC of four-fold to 1 μg mL^{-1} (Table 1). Gentamicin, another aminoglycoside, has also been shown to be an effective treatment option for patients suffering from tularemia.[20] Screening the same library for gentamicin repotentiation gave the same results for streptomycin, with compound **1** lowering the MIC four-fold from 1 μg mL^{-1} to 0.125 μg mL^{-1}.

Another class of antibiotics tested were macrolides, specifically azithromycin. Azithromycin is an attractive option for treatment because of its ability to concentrate within macrophages, where Francisella replicates at intracellular levels that are even greater than serum levels.[21] Upon screening our library, compound **1** exerted no change in azithromycin activity. Another compound (**2**) displayed an eight-fold reduction in MIC, from 4 μg mL^{-1} to 0.5 μg mL^{-1}). Compound **2** is of interest because unlike previously reported small molecule adjuvants from our lab it is neither a 2-AI nor 2-ABI, but rather contains an indoline core.

Our screen was further expanded to include a phosphonic acid antibiotic, FR900098. FR900098 has been shown to be effective at inhibiting *Francisella* sub-species.[22] Compound **3** displayed a significant decrease in MIC of 16-fold from 1024 μg mL^{-1} to <64 μg mL^{-1}.

Finally, we used colistin, a polymyxin antibiotic that is known to be ineffective at treating *Francisella* infections due to intrinsic resistance.[23] The MIC of colistin alone was 256 μg mL^{-1}. Library screening revealed an indole containing compound (**4**) that was also absent of a 2-AI or 2-ABI motif. The indole **4** exhibited a four-fold MIC reduction for colistin to 64 μg mL^{-1}.

Library synthesis for structure activity relationship (SAR) study

Based on the results of our screen, availability of starting materials and cost of antibiotics, we opted to perform analogue synthesis of the indole **4**. We were able to rapidly assemble an 11-membered analogue library of **4** (ESI† Table S1). The core is structurally similar to tryptamine with the only difference being a shorter 2-carbon linker. Direct reaction of tryptamine and derivatives with a variety of acylating agents in the presence of TEA gave a library of various structural motifs. Testing revealed that shortening the three-carbon linker to two carbons (**5**) drastically increased potency (Scheme 1), lowering the MIC of colistin 32-fold to 8 μg mL^{-1}. The amide appears to be necessary as any change in functionality: amine, ester, or sulfonamide, results in complete loss in activity (ESI† Table S1). Alkylation or acylation of the indole nitrogen-1 position, also caused complete loss in MIC suppression. Changes in the alkyl tail length from butyl also gave disappointing results, as methyl, ethyl, and propyl derivatives had no impact on the antibiotic MIC.

Time kill curve of compound 5

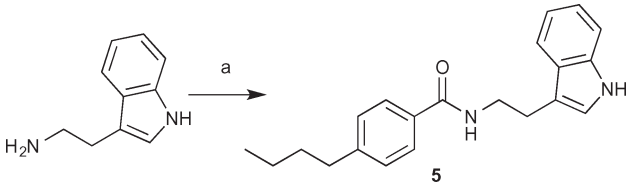
In order to determine whether compound **5** was acting through a toxic or non-toxic mechanism, bacterial growth was measured as a function of time. *F. philomiragia* was grown with/without compound (50 μM) being present. Bacterial growth was checked at 8, 12, 16, 20, and 24 h time points (Fig. 2). Based on the analysis of the time kill curve, we observe minor growth delay at earlier time points; however growth is identical by 16 hours.

MIC determination in *Francisella novicida*

With lead compounds in hand, we then wanted to determine whether compounds retained activity against *F. novicida*, a sub-species of *F. tularensis*. As before, the MICs of the active compounds (**1**, **2**, and **5**) were determined and then antibiotic MICs were established in the absence or presence of each compound at 25% MIC. Compound **1** showed no change in MIC for any of the antibiotics tested (Table 2). The indole compound **5** displayed a two-fold reduction in MIC for colistin from 1024 μg mL^{-1} to 512 μg mL^{-1}. The concentration of **5** was increased by 10 μM increments from 50 μM to 100 μM;

Table 1 MIC data for the initial lead compounds against *F. philomiragia*. ND = not determined

Compound	MIC (μM)	Concentration tested (μM)	Streptomycin (μg mL^{-1})	Gentamicin (μg mL^{-1})	Azithromycin (μg mL^{-1})	FR900098 (μg mL^{-1})	Colistin (μg mL^{-1})
—	—	—	4	0.5	4	1024	256
1	>200	50	1	0.125	4	1024	32
2	>200	50	4	0.5	0.5	1024	256
3	200	50	8	1	2	<64	512
4	>200	50	4	0.25	2	ND	64
5	>200	50	4	0.25	0.5	1024	8



Scheme 1 Synthesis of the most active compound (**5**). Reagents and conditions: (a) 4-butylbenzoyl chloride, TEA and DCM.

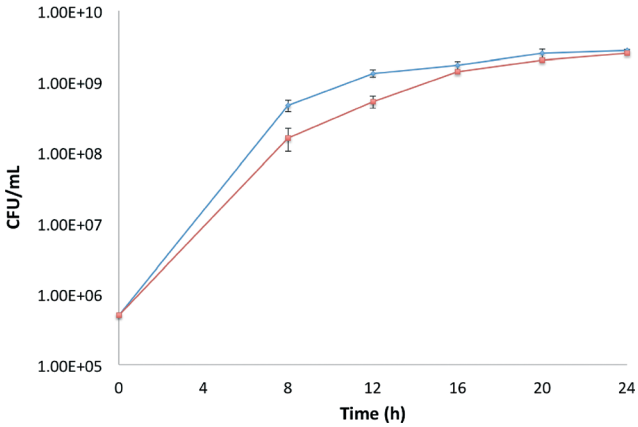


Fig. 2 Time kill curve of compound **5**. Blue diamonds represent bacterial control. Red squares represent bacteria and compound **5** (50 μM).

however the increased concentration of **5** was unable to potentiate colistin in *F. novicida* further. Compound **2** was the only adjuvant tested to show any significant activity, reducing the MIC of azithromycin 16-fold fro 2 μg mL^{-1} to 0.125 μg mL^{-1}.

Experimental section

Materials and methods

All reagents and solvents for synthesis were obtained from Sigma-Aldrich, St. Louis, MO, USA. Triethylamine was dried by refluxing CaH_2, followed by distillation and storage over 4 Å molecular sieves. Deuterated solvents were acquired from Cambridge Isotope Laboratories (CIL). Purification was performed using 60 mesh standard silica from Sorbtech. ^1H NMR (300 MHz) and ^{13}C NMR (100 MHz) spectra were recorded at 25 °C on Varian Mercury spectrometers. Chemical shifts (δ) are given in ppm relative to TMS as an internal standard or the respective NMR solvent. Mass spectra were recorded on Thermo Fisher Scientific Exactive Plus MS (ESI).

Synthesis

General procedure for amide, ester, and sulfonamide coupling. In a flame dried round bottom under N_2 atmosphere was added tryptamine (300 mg, 1.87 mmol), TEA (777 μL, 5.61 mmol), and anhydrous DCM (10 mL). The reaction mixture was cooled to 0 °C and 4-butylbenzoyl chloride (314 μL, 1.68 mmol) was added dropwise. The reaction mixture was stirred until completion as determined by TLC. The reaction mixture was then washed with 1 N HCl (1 × 5 mL), brine (1 × 5 mL) and the organic layer was dried over Na_2SO_4, then filtered. The solvent was removed *in vacuo* and the residue was purified by flash chromatography using 4 : 1 Hex/EtOAc to 2 : 1 Hex/EtOAc to give a white solid (538 mg, 93% yield).

Biological

Bacteria, media, and antibiotics. *Francisella philomiragia* was obtained from the American Type Culture Collection (ATCC), Manassas, VA, as ATCC # (25015) and *Francisella novicida* was obtained from BEI Resources as BEI # (U112). *F. philomiragia* and *F. novicida* were grown in cation adjusted Mueller Hinton broth (CAMHB) supplemented with 2% IsoVitaleX (BD #211876) enrichment or tryptic soy broth supplemented with 0.1% cysteine (TSB-C) at 37 °C with shaking, or on cysteine heart agar supplemented with 5% rabbits blood (Hemostat; DRB050) at 37 °C. The bacteria were incubated at 37 °C for 24 h. Antibiotics were purchased from Sigma-Aldrich, St. Louis, MO, USA.

Minimum inhibitory concentration (MIC) assay. One day cultures (24 h) were subcultured to 5 × 10^5 CFU mL^{-1} in CAMHB supplemented with 2% IsoVitaleX (*F. philomiragia*) or TSB-C (*F. novicida*). Aliquots (1 mL) of the subcultured media were added to small culture tubes. Compound from a 100 mM stock solution in DMSO was added to the small culture tube to give the desired starting concentration (200 μM or 2–2048 μg mL^{-1}). Rows 2–12 of a 96-well microtiter plate were filled with 100 μL per well of the bacterial subculture. The wells of the first row were filled with 200 μL each of the compound-containing sample. Row 1 wells were mixed a minimum of five times, followed by a 100 μL aliquot to row 2. The process was repeated until the final row, in which the last 100 μL aliquot was discarded. The 96-well plate was then sealed with a plastic lid and incubated under stationary conditions at 37 °C for 48 h. The compound MIC was recorded as the lowest concentration at which no bacterial growth was observed.

Antibiotic resensitization assay. One day cultures (24 h) were subcultured to 5 × 10^5 CFU mL^{-1} in CAMHB supplemented with 2% IsoVitaleX (*F. philomiragia*) or TSB-C

Table 2 MIC data for active compounds against *F. novicida*

Compound	MIC (μM)	Concentration tested (μM)	Gentamicin (μg mL^{-1})	Azithromycin (μg mL^{-1})	Colistin (μg mL^{-1})
—	—	—	1	2	1024
1	>200	50	1	2	1024
2	>200	50	4	0.125	1024
5	>200	50	4	1	512

(*F. novicida*). Aliquots (5 mL) of subcultured media were added to large culture tubes. Compound from a 100 mM stock solution in DMSO was added to the large culture tubes to give the concentration to be tested (25% of the MIC value). One culture tube had nothing added and served as the control. Aliquots (1 mL) were taken from the large culture tubes and added to small culture tubes. Antibiotic was added to the small culture tubes, including the control, to give the desired starting concentration (2–2048 μg mL^{-1}). Rows 2–12 of a 96-well microtiter plate were filled with 100 μL per well of the bacterial subculture with or without compound. The wells of the first row were filled with 200 μL each of the antibiotic dosed samples. Row 1 wells were mixed a minimum of five times, followed by a 100 μL aliquot to the subsequent row. The process was repeated until the final row, in which the last 100 μL aliquot was discarded. The 96-well plate was then sealed with a plastic lid and incubated under stationary conditions at 37 °C for 48 h. The compound MIC was recorded as the lowest concentration at which no bacterial growth was observed.

Time kill curve. *F. philomiragia* was cultured for 24 h in CAMHB supplemented with 2% IsoVitaleX. Fresh media was then inoculated with the bacteria to 5×10^5 CFU mL^{-1} and aliquots (3 mL) were added to large culture tubes. Compound (25% of the MIC) was added to one set of culture tubes, while the control was bacteria alone. The tubes were incubated at 37 °C with shaking. Aliquots (100 μL) were taken at 8, 12, 16, 20, and 24 h time points. The aliquots were serially diluted and plated on cysteine heart agar supplemented with 5% rabbit's blood and spread with sterile glass beads. Plates were incubated at 37 °C under stationary conditions for 24–36 h. The number of colonies formed were enumerated.

Conclusions

In summary, a pilot library screen resulted in the identification of several unique small molecule adjuvants, capable of potentiating various classes of antibiotics against *F. philomiragia*. Analogue synthesis of one lead (**4**) gave rise to compound **5**, which displayed enhanced activity, culminating in a 32-fold MIC reduction to 8 μg mL^{-1} for colistin. Bacterial growth over time was measured to elucidate whether compound **5** was bactericidal. The CFUs measured for compound containing samples, correlate strongly with the control providing evidence **5** is acting through a non-toxic mechanism. This is the first example of a small molecule adjuvant able to potentiate *F. philomiragia* resistance to colistin, and **5** may represent the basis of a new class of small molecule adjuvants. Further exploration of this unique class of compound is currently underway in attempts to break *F. novicida* resistance.

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