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Using Small-Molecule Adjuvants to Repurpose Azithromycin for Use against *Pseudomonas aeruginosa*

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Abstract

A major contributor to fatalities in cystic fibrosis (CF) patients stems from infection with opportunistic bacterium *Pseudomonas aeruginosa*. As a result of the CF patient's vulnerability to bacterial infections, one of the main treatment focuses is antibiotic therapy. However, the highly adaptive nature of *P. aeruginosa*, in addition to the intrinsic resistance to many antibiotics exhibited by most Gram-negative bacteria, means that multi-drug-resistant (MDR) strains are increasingly prevalent. This makes the eradication of pseudomonal lung infections nearly impossible once the infection becomes chronic. New methods to treat pseudomonal infections are greatly needed in order to eradicate MDR bacteria found within the respiratory tract, and ultimately better the quality of life for CF patients. Herein, we describe a novel approach to combatting pseudomonal infections through the use of bis-2-aminoimidazole adjuvants that can potentiate the activity of a macrolide antibiotic commonly prescribed to CF patients as an anti-inflammatory agent. Our lead bis-2-AI exhibits a 1024-fold reduction in the minimum inhibitory concentration of azithromycin in vitro and displays activity in a *Galleria mellonella* model of infection.

Graphical Abstract

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Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: [10.1021/acsinfecdis.8b00288](https://doi.org/10.1021/acsinfecdis.8b00288).

Representative list of compounds screened; azithromycin potentiation data against PAO1; PAO1 membrane permeabilization data using the BacLight assay; mitigating effects of EDTA with compound 2; azithromycin MIC potentiation of compound 2 in minimal salts media; PAO1 membrane permeabilization data with divalent cation metals; azithromycin potentiation data against KP2146; KP2146 membrane permeabilization data using the BacLight assay; detergent Tween 80 results; hemolysis results; *Galleria mellonella* results; references for initial compound screening; and representative ¹H and ¹³C NMR spectra (PDF)

The authors declare the following competing financial interest(s): Dr. Melander is cofounder of Agile Sciences, a biotechnology company seeking to commercialize antibiotic adjuvants.

Keywords

antibiotic resistance; antibiotic adjuvant; cystic fibrosis; *Pseudomonas aeruginosa*

Cystic fibrosis (CF) is a life-threatening genetic disease inherited by approximately 1 in 2000 children annually within the American Caucasian population alone.¹ The Cystic Fibrosis Foundation estimates that approximately 70 000 people suffer with CF worldwide.² CF is defined by a defect in the cystic *fibrosis transmembrane conductance regulator* (*CFTR*) gene, which causes the overaccumulation of thick, sticky mucus that coats the lungs and intestines while acting as an ideal breeding ground for bacterial infections.³ Pulmonary failure as a result of prolonged inflammation is directly associated with the presence of *Pseudomonas aeruginosa* and remains the main cause of death in CF patients.⁴ The ongoing persistence of *P. aeruginosa* within the CF community is due in part to its highly adaptive defense mechanisms and the lack of novel, effective antimicrobial drugs to treat multi-drug-resistant (MDR) pseudomonal strains. The average age at which a CF patient acquires a *P. aeruginosa* infection is approximately 16 years, and once physicians observe a change in pseudomonal cells from nonmucoid to mucoid, the life expectancy of each patient is soon established, with the average being 37 years.^{1,2}

Treating MDR *P. aeruginosa* is challenging and requires aggressive action, typically via the administration of a combination of two or three antimicrobials. Some of the commonly used antipseudomonal drugs include aminoglycosides, carbapenems, cephalosporins, fluoroquinolones, and polymyxins.⁵ Of grave concern is that acquired resistance mechanisms toward all of these antibiotic classes have been found in numerous strains of MDR *P. aeruginosa*.⁶ This is especially troubling in the case of colistin, as it is a last-resort antibiotic prescribed to CF patients in Europe. Colistin, however, has not been approved as a CF treatment option within the United States because of its reported nephro- and

neurotoxicity.^{7,8} Novel treatment approaches to combatting pseudomonal infections are clearly needed.

In addition to antibiotic therapy, anti-inflammatory drugs are administered to CF patients as a life-long regimen in attempts to increase patient comfort and expand life expectancy.⁹ Azithromycin is a macrolide antibiotic that has demonstrated the ability to reduce tissue inflammation in several lung-associated diseases and is currently prescribed to CF patients as the main anti-inflammatory drug.^{9–12} Outside of CF treatment, azithromycin is used as an antibiotic to treat infections caused by Gram-positive pathogens, such as *Staphylococcus aureus*.¹³ However, like all macrolide antibiotics, azithromycin does not possess the ability to combat *P. aeruginosa* infections due to the intrinsic resistance imparted by both the outer membrane and efflux pump systems.¹⁴

Previously, we have demonstrated that select members of our in-house marine-alkaloid-derived library possess adjuvant activity and can break the acquired resistance toward ceftazidime and meropenem in planktonic MDR *P. aeruginosa*.¹⁵ Other groups have shown that cationic peptides enhance the activity of azithromycin against Gram-negative pathogens in a synergistic manner through perturbing the outer membrane and thus allowing antibiotics such as azithromycin entry into the cell.^{16,17} Given azithromycin's established use in CF therapy, we wondered whether we could identify an adjuvant from our in-house library of nitrogen-dense hetero-cycles that would break *P. aeruginosa*'s intrinsic resistance to azithromycin and in the process allow us to repurpose azithromycin for antibacterial use against this pathogen. Herein, we describe the identification of nonmicrobicidal bis-2-aminoimidazoles (bis-2-AIs) **1** and **2** that potentiate azithromycin activity in highly resistant strains of *P. aeruginosa*. Furthermore, we demonstrate the requirement for the bis-2-AI moiety compared to a mono-2-AI analog through structure–activity relationship (SAR) studies. After identifying a lead bis-2-AI adjuvant, we sought to identify the potential mechanism of action by studying its effect on bacterial membrane integrity and efflux. Next, we evaluated and confirmed the in vivo activity of a combination of bis-2-AI **2** and azithromycin in a *Galleria mellonella* model of infection. We then demonstrated that bis-2-AI **2** is able to sensitize PAO1 to an additional macrolide antibiotic, clarithromycin, as well as other classes of antibiotics. Finally, we established the ability of adjuvant **2** to potentiate azithromycin activity against several additional clinically relevant *P. aeruginosa* strains isolated from CF patients.

RESULTS AND DISCUSSION

This study was initiated by screening a structurally diverse subset of compounds from our internal library for the ability to lower the minimum inhibitory concentration (MIC) of azithromycin against *P. aeruginosa* laboratory strain PAO1, which exhibits high levels of resistance to azithromycin (MIC 256 $\mu\text{g/mL}$). (See the Supporting Information for a representative list of screened compounds.) From this screen, compound **1**, which possesses two 2-AI groups, lowered the MIC of azithromycin by 8-fold, from 256 to 32 $\mu\text{g/mL}$ at 60 μM . Prompted by this result, structurally similar compounds **2** and **3** were screened against PAO1 (also at 60 μM). Bis-2-AI **2** exhibited potent adjuvant activity, lowering the MIC of azithromycin 1024-fold from 256 to 0.25 $\mu\text{g/mL}$. Interestingly, bis-2-AI **3** did not sensitize

PAO1 to azithromycin, inspiring us to explore the structure–activity relationship (SAR) of these bis-2-AI analogs.

Using lead adjuvant **2** as an inspiration for SAR studies, a pilot library was synthesized for the direct comparison of structure and biological activity (Schemes 1 and 2). Beginning with amino acid methyl esters **4** and **5**, 2-AI analogs **6** and **7** were synthesized using a previously published procedure, which utilized standard Akabori reduction conditions followed by the condensation of cyanamide in a temperature- and pH-controlled environment (Scheme 1A).¹⁸ The pH of the cyanamide condensation reaction was previously determined to be of importance by Lancini and Lazzari¹⁹ to avoid the production of urea in highly acidic environments or undesired aminoketone formations in neutral or alkaline environments. For SAR purposes, 2-AI derivatives **10** and **13** were accessed using commercially available carboxylic acid **8** or acid chloride **11**. After the conversion of compound **8** into its 6-azido acid chloride congener, this and **11** were subjected to reaction with diazomethane followed by a HBr workup to produce the corresponding α -bromoketones (Scheme 1B,C). These intermediates were subsequently cyclized with Boc-guanidine to produce compounds **9** and **12**. Upon reduction of the azide moiety of compound **9** and deprotection of the 2-AIs, syntheses of compounds **10** and **13** were completed by conversion to their corresponding HCl salts for biological testing.

Bis-2-AI derivatives were then accessed via an oxidative dimerization route developed by the Horne group.²⁰ Compounds **14** and **15** were previously prepared in our group.²¹ The treatment of each 2-AI compound with 1 equiv of *N*-chlorosuccinimide under acidic conditions followed by reaction with commercially available 2-AI sulfate generated bis-2-AIs **1**, **2**, and **16–19** as well as tricyclic compounds **20** and **21** as low-yielding byproducts (Scheme 2).

The MIC of each bis-2-AI was first determined, and all active adjuvants returned MICs 200 μ M, with the exception of compound **19**, which exhibited an MIC of 100 μ M (select data summarized in Table 1). The MIC of azithromycin in the presence of each compound at 60 μ M (30 μ M for compound **19**) was next determined. The mono-2-AI intermediates, **6**, **7**, **10**, **13**, and **14**, did not enhance the azithromycin activity, suggesting the importance of a second 2-AI functional group for activity (Table S2). In addition, tricyclic compounds **20** and **21** containing two 2-AI heads tethered by a seven-membered ring also lacked the ability to potentiate azithromycin, implying the significance of the two 2-AI heads being in close proximity to one another while retaining conformational flexibility (Table S2).

Considering the highly polar nature of active bis-2-AI analogs, we evaluated the biological importance of polarity by synthesizing and screening bis-2-AIs **17–19**, which possess aliphatic tails (4, 13, or 15 carbons, respectively) lacking a positively charged amine in this region of the molecule. All analogues failed to sensitize PAO1 to azithromycin; however, bis-2-AI **19** did exhibit greater toxicity against PAO1 alone. The fact that adjuvants **17–19** failed to sensitize PAO1 to azithromycin led to the conclusion that the presence of a free amine in the distal tail region of the molecule is crucial to maintaining activity (Table S2).

Finally, to delineate the ideal length of the aliphatic tail for amine-containing bis-2-AIs, the synthesis of bis-2-AI **16** was carried out. Bis-2-AI **16** did modestly potentiate azithromycin activity by 16-fold, similar to that of bis-2-AI **1** (Table 1). With this knowledge, it was revealed that compound **2**, which contains a four-carbon alkyl chain, remained the most active compound in these studies. To continue the investigation of lead bis-2-AI **2** activity, a dose–response study was performed against PAO1 over a wide range of concentrations (Table 2). This revealed substantial adjuvant activity at 40 μ M, where there was a 256-fold reduction in the MIC of azithromycin before a drop-off in activity was observed at lower adjuvant concentrations.

Next, we wanted to probe the mechanistic basis by which compound **2** is able to sensitize PAO1 toward azithromycin. Gram-negative species are far more challenging to combat than Gram-positive species, predominantly due to the protection imparted in the form of the outer membrane.²² Therefore, we first investigated whether compound **2** was acting by increasing the permeability of the bacterial cell membrane using a commercially available LIVE/DEAD BacLight Bacterial Viability Kit. The BacLight assay quantifies the fluorescence of two DNA-binding dyes to measure the ratio of intact/damaged cells. Lead compound **2** was tested in addition to both compounds **1** and **3** for comparison. PAO1 cells were treated with each compound at 60 μ M for 1 h, after which cells were collected and washed and the fluorescence was determined. The degree of membrane permeability imparted by compound **1** was not as substantial as that of compound **2**, while compound **3** had a negligible effect, indicating a correlation between membrane permeability and adjuvant activity. Exposure to compound **2** resulted in an increase in the permeability to 50% compared to that of untreated cells, while exposure of compound **3** resulted in only a 1% increase in membrane permeability (Table S3). Furthermore, compound **1**, which effected a more modest reduction in the MIC of azithromycin than compound **2**, caused a 31% increase in membrane permeability in PAO1 (Table S3). These results suggested that increasing the cell membrane permeability is correlated to adjuvant activity and may play a role in the mechanism by which bis-2-AI **2** enhances azithromycin activity.

Bis-2-AI adjuvants **1** and **2** were also screened for the potentiation of azithromycin against another important Gram-negative pathogen, *Klebsiella pneumoniae*. Neither bis-2-AI adjuvant **1** or **2** potentiated azithromycin activity against *K. pneumoniae* ATCC BAA-2146, effecting only a 2-fold or less reduction in the azithromycin MIC (Table S4). Compound **3** was likewise tested for azithromycin potentiation activity against *K. pneumoniae* BAA-2146 and resulted in merely a 2-fold MIC reduction (Table S4). In order to further probe the role of disruption of the integrity of the cell membrane in bis-2-AI activity, a BacLight assay was performed using *K. pneumoniae* BAA-2146, which revealed a 5% increase in cell membrane permeability after treatment with compound **2**, considerably less than the 50% increase observed for PAO1 at the same concentration (Table S5). This further indicates that an increase in cell membrane permeability is correlated with adjuvant activity, although it is not yet known whether this is direct or indirect.

Many bacterial species, including *P. aeruginosa*,²³ depend on biological metals obtained from the human host to maintain metabolism and cell growth as well as contribute to bacterial virulence.²⁴ As divalent metal ions are also important for eukaryotic cell growth

and metabolism, one approach implemented by the host during infection is to sequester free metals from the environment using host proteins, such as transferrin, in order to reduce metal availability for invading pathogens.²⁵ Therefore, metal deprivation, also known as “nutritional immunity”, is a crucial defense mechanism used by the innate immune system to essentially starve microbial pathogens.²⁶ *P. aeruginosa* utilizes several strategies to circumvent nutritional immunity, such as pirating metal ions from host resources (e.g., transferrin) using siderophores or scavenging free metal ions from the environment and transporting them into the cell via several well-studied mechanisms.²⁴ Previous studies within our laboratory have shown that 2-aminobenzimidazole (2-ABI) small-molecule adjuvants that inhibit and disperse Gram-positive biofilms do so via a zinc-dependent mechanism.^{27,28} When analyzing the structure of these bis-2-AIs, we recognized that these molecules could be acting as metal chelators. We therefore measured the effect of increasing concentrations of iron and other commonly encountered divalent metal cations on the ability of compound **2** (at 60 μ M) to potentiate azithromycin against PAO1 (Table 3).

An increasing concentration of Fe(II) or Mn(II) suppressed the ability of compound **2** to potentiate azithromycin. When supplemented with 200 μ M FeSO₄·7H₂O, the activity of compound **2** was negligible, and supplementation with 200 μ M MnCl₂ also caused a reduction in activity (a 16-fold reduction in azithromycin MIC compared to 1024-fold). However, doping with Zn(II), Ca(II), Cu(II), or Mg(II) did not affect the activity of adjuvant **2**. Prompted by the effects of iron and manganese on bis-2-AI activity, we conducted additional studies to evaluate the mechanism of action of bis-2-AI **2** against PAO1.

To further probe the mechanism of action of bis-2-AI **2** and the effect that metal ions elicited on activity, we wanted to analyze compound **2**'s ability to potentiate azithromycin in a chelating-competitive environment by codosing with ethylenediaminetetraacetic acid (EDTA). Many studies have shown that EDTA can displace divalent cation cross-bridges necessary for *P. aeruginosa* membrane integrity, and it has been used in combination with antimicrobials to combat Gram-negative bacterial pathogens.^{29,30} EDTA chelates positively charged ions, thus codosing with EDTA would sequester any Fe(II) from the media and allow an observation of the activity of compound **2** in the absence of Fe(II). A dose-dependent study was performed against PAO1 in which the ability of compound **2** to potentiate azithromycin was measured in increasing concentrations of EDTA (Table S6). The supplementation of EDTA at high concentrations (200 μ M) did not exhibit any biological activity on PAO1 when dosed alone nor did it alter the MIC of azithromycin against PAO1 (Table S6). Furthermore, the triple dosing of EDTA at increasingly high concentrations (up to 200 μ M) of adjuvant **2** and azithromycin did not alter the previously observed adjuvant activity of **2**, with a 1024-fold reduction in the azithromycin MIC noted.

To further validate that bis-2-AI **2** adjuvant activity is not due to metal chelation, the azithromycin potentiation assay was performed in minimal salts media (M9 media). Cation-adjusted Mueller-Hinton broth (CAMHB) is typically the medium used for MIC determination against *P. aeruginosa* according to the Clinical and Laboratory Standards Institute (CLSI).³¹ It has been reported that divalent ions can affect the mode of action of several antimicrobial agents and the stabilization of cell walls; therefore, the supplementation of ions in CAMHB has been the standardized medium for accurate

antimicrobial results against PAO1.³² However, we wanted to observe the activity of adjuvant **2** in iron-deficient media, such as M9. We first established the MICs of azithromycin and compound **2** against PAO1 in M9 media (Table S7). As expected, the MICs of azithromycin and compound **2** were consistent with their MICs against PAO1 in CAMHB, being 256 $\mu\text{g/mL}$ and 200 μM , respectively, suggesting that the potency of azithromycin against PAO1 is not affected when in an iron-deprived environment, which is in agreement with the EDTA experiment. Likewise, compound **2** exhibits a comparable potentiation of azithromycin against PAO1 in M9 as in CAMHB media, lowering the MIC 512-fold from 256 to 0.5 $\mu\text{g/mL}$.

Prompted by the effects of high concentrations of both Fe(II) and Mn(II) on the potentiation activity, the effect of compound **2** on PAO1 membrane integrity in the presence of 200 μM Fe(II) and Mn(II) was evaluated again using the BacLight assay. We first analyzed the effect of Fe(II) and Mn(II) on PAO1 membrane integrity before combining a metal and adjuvant against PAO1, and we observed that neither metal ion affected the permeability when administered alone. (Table S8). The presence of Fe(II) at 200 μM significantly reduced the effect of compound **2** (at 60 μM) upon PAO1 membrane permeability, resulting in only a 14% increase in membrane permeability compared 50% in the absence of Fe(II) (Table S8). Furthermore, the presence of Mn(II) at 200 μM also reduced the increase in PAO1 membrane permeability effected by compound **2** to just 8%.

Next, we wanted to exclude the possibility that bis-2-AI **2** acts through a nonspecific aggregation-based (i.e., PAINS) mechanism.³³ To address this issue, we conducted the antibiotic potentiation assay in the presence of a detergent, Tween 80. Bis-2-AI **2** adjuvant activity was unaffected in the presence of 0.01% Tween 80, indicating that adjuvant activity is not driven by compound aggregation (Table S9).

To analyze eukaryotic toxicity, we measured the hemolytic activity of bis-2-AI compounds **2** and **3** upon defibrinated sheep's blood.³⁴ Both highly active compound **2** and inactive bis-2-AI **3** exhibited less than 3% hemolysis at concentrations of up to 500 μM , a concentration well above the adjuvant activity values (Table S10). We also analyzed the toxicity of our lead bis-2-AI **2** in a whole organism study using *Galleria mellonella* and observed 100% worm survival after 6 days following dosing with compound **2** at a high concentration of 400 mg/kg (Table S16).^{34,35} Both the hemolysis assay and *Galleria* studies suggest that bis-2-AI **2** does not exhibit significant eukaryotic toxicity.

Given the favorable eukaryotic toxicity profile, we next explored the ability of adjuvant **2** to potentiate azithromycin against PAO1 in a *G. mellonella* model of infection, which has been shown to be predictive of activity in murine studies.³⁵ We first identified meropenem as a positive control for PAO1 infections in the worm model. In *G. mellonella*, 30 min after worms were inoculated with 10^5 CFU PAO1, a single dose of meropenem at 5 mg/kg resulted in a 100% survival rate after 6 days (Table S17). We then attempted to determine the dosage of azithromycin necessary for minimal protection against infection; however, azithromycin dosed at concentrations of up to 100 mg/kg provided no survival (Table S12 and S13). Because azithromycin is not used as an antibiotic for *P. aeruginosa* and we did not want to surpass toxic levels, we decided to continue with combinatorial treatments by

modeling our treatment dosage after previously published work and to treat worms with azithromycin at 50 mg/kg.^{35–37} The treatment of infected worms with bis-2-AI **2** at 50 mg/kg provided no improvement in survival rates compared to those of untreated, infected worms (Table S14). However, a single dose of compound **2** at 50 mg/kg in combination with 50 mg/kg azithromycin improved the survival rate to 43% (Figure 2 and Table S15). This survival rate is equivalent to the treatment of infected worms with clavulanic acid/penicillin, one of the few clinically approved antibiotic/adjuvant combinations.³⁶

Following promising model organism results for lead bis-2-AI **2**, its potential as an adjuvant for additional antibiotics other than azithromycin was investigated against PAO1 (Table 4). Compound **2** (at 60 μ M) was able to substantially sensitize PAO1 to another macrolide antibiotic, clarithromycin, decreasing the MIC 128-fold from 256 to 2 μ g/mL. Bis-2-AI **2** was also able to sensitize PAO1 to rifampin, an antibiotic prescribed to patients with lung-associated disease tuberculosis.³⁸ Additionally, adjuvant **2** potentiated the activity of the β -lactam antibiotic doripenem, lowering the MIC 32-fold. The activity of colistin, another antibiotic that possesses activity against *P. aeruginosa*, was not enhanced by bis-2-AI **2**. It is interesting that bis-2-AI **2** did not sensitize *P. aeruginosa* to colistin, which elicits its antibacterial activity by binding to the outer cell membrane of Gram-negative bacteria and promoting its own uptake⁶ yet was able to sensitize PAO1 to doripenem and rifampin, both of which must diffuse across the cell membrane to produce their antibiotic effect on the bacterium.^{38,39} The presence or lack of adjuvant activity of compound **2** across this range of classes of antibiotics correlates to the data obtained in the BacLight analysis, indicating that compound **2** is affecting the outer membrane of the bacterium.

Finally, we evaluated the adjuvant potential of bis-2-AI **2** upon azithromycin activity against a panel of pseudomonal strains that are clinically relevant to CF by obtaining six isolates from CF patients, three of which are mucoidal and three of which are nonmucoidal isolates (Table 5). Bis-2-AI **2** caused 512-, 32-, and 128-fold reductions in the azithromycin MIC against three nonmucoid strains CEC 45, 57, and 102, respectively. A similar pattern of activity was observed in two of the three mucoid strains, where 32- and 16-fold reductions in the MIC were observed against CEC 103 and 111, respectively. A substantial increase in adjuvant activity was revealed against the third mucoid strain, CEC 70, for which a 4267-fold reduction in the azithromycin MIC was recorded. These results confirmed adjuvant **2**'s viability in the range of *P. aeruginosa* strains that are found among CF patients.

In conclusion, there is no question that MDR *P. aeruginosa* infections have devastated the quality of life and diminished the life expectancy for CF patients. Novel approaches to eradicate pseudomonal infections are greatly needed. We report a novel adjuvant containing a bis-2-AI moiety that potentiates the activity of a nonpseudomonal antibiotic, azithromycin, against a highly intrinsically resistant strain of *P. aeruginosa*, PAO1, as well as six CF clinical pseudomonal isolates. SAR studies revealed bis-2-AI **2** as the lead adjuvant, which enhances azithromycin activity by 1024-fold against PAO1 without displaying signs of hemolytic activity or *G. mellonella* toxicity well above the active concentration. Bis-2-AI **2** sensitized PAO1 to additional antibiotics including another macrolide antibiotic, clarithromycin, a β -lactam antibiotic doripenem, as well as rifampin but did not potentiate the activity of membrane-active antibiotic colistin. Interestingly, all of the antimicrobials that

were potentiated by bis-2-AI **2** are known to require penetration through the outer membrane in order to be effective. We hypothesize that the ability of compound **2** to enhance macrolide activity in *P. aeruginosa* is somehow related to its effect on membrane integrity, as seen in the BacLight assay; however, further mechanistic studies for compound **2** are currently ongoing to address whether this is due to a direct interaction with the membrane or an indirect effect due to altered cell membrane structure/presentation. Activity was impacted by both Fe and Mn ions; however, the activity of these compounds does not appear to be driven by chelation. Finally, nontoxic, nonmicrobicidal bis-2-AI **2** was able to potentiate azithromycin in a model organism, exhibiting a 43% survival rate for worms inoculated with PAO1, similar to that reported for clavulanic acid/penicillin.³⁶ Because the *G. mellonella* model has inherent limitation, specifically, it is limited to a single dose, we expect that moving to a more clinically relevant model where multiple doses are given will return significantly greater survival.

EXPERIMENTAL SECTION

All reagents used for chemical synthesis were purchased from commercially available sources and used without further purification. Flash chromatography was performed using a 60 Å mesh standard-grade silica gel from Sorbetch. NMR solvents were obtained from Cambridge Isotope Laboratories and used as received. All ¹H NMR spectra (300, 400, or 600 MHz) were recorded at 25 °C on Varian Mercury spectrometers or a (700 MHz) Bruker Avance spectrometer. All ¹³C NMR (100, 125, or 175 MHz) spectra were recorded at 25 °C on Varian Mercury spectrometers. Chemical shifts (δ) are given in parts per million (ppm) relative to the respective NMR solvent; coupling constants (*J*) are in hertz (Hz).

Abbreviations used are s, singlet; bs, broad singlet; d, doublet; dd, doublet of doublets; t, triplet; dt, doublet of triplets; and m, multiplet. All high-resolution mass spectrometry measurements were made in the Molecular Education, Technology, and Research Innovation Center (METRIC) at North Carolina State University. The purities of the tested compounds were all verified to be >95% by LC-MS analysis on a Shimadzu LC-MS 2020 with a Kinetex, 2.6 mm, C18 50 × 2.10 mm column.

General Procedure for Akabori Reduction and Cyanamide Cyclization (**6** and **7**).

Commercially available amino acid methyl ester derivatives **4** and **5** (54 mmol, 1 equiv) were dissolved in deionized water (125 mL), and 250 g of 5% sodium amalgam was added over 1 h. The pH was maintained at 1.5 by the addition of concentrated HCl, and the temperature was kept at 0 °C using an ice bath. The reaction mixture was stirred at 0 °C for an additional 30 min, whereupon the mercury was separated. To the resulting solution was added cyanamide (540 mmol, 10 equiv), and the pH was adjusted to 4.3 by the addition of 1 M NaOH. The solution was heated to 95 °C and stirred for 3.5 h and then evaporated to dryness under reduced pressure. The residue was washed in Et₂O to remove the unreacted cyanamide, dissolved in a minimal amount of MeOH and decanted to remove NaCl, and then concentrated under reduced pressure. The residue was purified by flash chromatography (25–100% MeOH saturated with NH₃ in DCM). Purified product was dissolved in 6 M HCl in MeOH (10 mL), and the solvent was removed to yield **6** or **7** as its HCl salt.

General Procedure for Oxidative Heterodimerization (1, 2, and 16–21).

NCS (2 mmol, 1 equiv) was added to a solution of the corresponding 2-AI derivative (2 mmol, 1 equiv) in methanesulfonic acid (8 mL) at room temperature. After 10 min, commercially purchased 2-AI sulfate (2 mmol, 1 equiv) was added, and the resulting solution was stirred for 48 h. The reaction mixture was then diluted with acetone and decanted (200 mL $\times$ 2). The resulting residue was purified using flash chromatography (25–100% MeOH saturated with NH_3 in DCM), the purified product was dissolved in 6 M HCl in MeOH, and the solvent was removed to yield the corresponding bis-2-AI **1**, **2**, and **16–21** as HCl salts.

General Procedure for Boc-guanidine Cyclization (9 and 12).

The corresponding α -bromoketone (2 mmol, 1 equiv) and Boc-guanidine (6 mmol, 3 equiv) were dissolved in anhydrous DMF (20 mL) and stirred for 48 h. The reaction mixture was diluted with deionized water, and the desired product was extracted with EtOAc (50 mL $\times$ 3). The organic fractions were combined and washed with brine (10 mL $\times$ 2), dried with MgSO_4 , and concentrated under reduced pressure. The residue was purified by flash chromatography (5–100% EtOAc/hexanes) to afford desired compounds **9** and **12**.

General Procedure for Boc Deprotection (10 and 13).

The corresponding Boc-protected 2-AI (1.3 mmol, 1 equiv) was dissolved in DCM (1 mL), and TFA (2 mL) was added to the reaction vessel dropwise. The reaction was stirred for 3 h, and then the solvent was removed under reduced pressure. The resulting TFA salt was dissolved in 6 M HCl in MeOH (10 mL), and the solvent was removed to yield **10** and **13** as HCl salts.

PREVIOUSLY REPORTED COMPOUNDS^{18,40,41}**4-(3-Aminopropyl)-1H-imidazol-2-amine (6).**

Compound **6** was prepared following a previously published protocol using the general procedure for the Akabori reduction and cyanamide cyclization to obtain compound **6** as a white solid in 41% yield. Spectral data for compound **6** was consistent with known values.⁴¹ ^1H NMR (300 MHz, CD_3OD) δ 6.61 (s, 1H), 2.98 (t, J = 7.8 Hz, 2H), 2.64 (t, J = 7.6 Hz, 2H), 2.06–1.90 (m, 2H).

4-(4-Aminobutyl)-1H-imidazol-2-amine (7).

Compound **7** was prepared following a previously published protocol using the general procedure for the Akabori reduction, and cyanamide cyclization was followed to afford compound **7** as an orange solid in 18% yield. Spectral data for compound **7** was consistent with known values.¹⁸ ^1H NMR (400 MHz, CD_3OD) δ 6.57 (s, 1H), 2.98 (t, J = 7.3 Hz, 2H), 2.57 (t, J = 6.6 Hz, 2H), 1.76–1.67 (m, 4H).

tert-Butyl 2-Amino-4-(5-azidopentyl)-1H-imidazole-1-carboxylate (9).

Compound **9** was prepared following a previously published protocol using the general procedure for Boc-guanidine cyclization to afford compound **9** as an orange solid in 33%

yield. Spectral data for compound **9** was consistent with known values.⁴⁰ ¹H NMR (300 MHz, CDCl₃) δ 6.50 (s, 1H), 5.63 (bs, 2H), 3.26 (t, J = 6.9 Hz, 2H), 2.36 (t, J = 7.3 Hz, 2H), 1.65–1.55 (m, 13H), 1.45–1.36 (m, 2H).

NOVEL COMPOUND CHARACTERIZATION

tert-Butyl 2-Amino-4-butyl-1*H*-imidazole-1-carboxylate (**12**).

Commercially available acid chloride **11** (17 mmol, 1 equiv) was dissolved in DCM (10 mL) and added dropwise to a solution of CH₂N₂ (51 mmol, 3 equiv) generated with Diazald/KOH in Et₂O (100 mL) and cooled to 0 °C. The reaction mixture was stirred for 1 h at 0 °C, whereupon concentrated HBr (4 mL) was added dropwise. The reaction mixture was stirred an additional 30 min at 0 °C and then quenched with saturated NaHCO₃(aq), and the organic material was extracted with Et₂O (100 mL $\times$ 2). The organic fractions were combined and washed with brine (20 mL $\times$ 2), dried with MgSO₄, and then concentrated under reduced pressure. The residue was purified by flash chromatography (5% EtOAc/hexanes) to afford its corresponding α -bromoketone, which was then used in the general procedure for Boc-guanidine cyclization to afford compound **12** as an orange solid in 47% yield. ¹H NMR (300 MHz, CD₃OD) δ 6.54 (s, 1H), 2.33 (t, J = 7.9 Hz, 2H), 1.60 (s, 9H), 1.53–1.49 (m, 2H), 1.39–1.32 (m, 2H), 0.93 (t, J = 7.3 Hz, 3H) ppm. ¹³C NMR (125 MHz, CD₃OD) δ 149.3, 138.2, 105.9, 84.6, 30.3, 27.1, 26.7, 22.0, 12.8 ppm. HRMS (ESI) calcd for C₁₂H₂₁N₃O₂ [M + H]⁺ 240.1707, found 240.1700.

4-Butyl-1*H*-imidazol-2-amine (**13**).

Using the general procedure for Boc deprotection, compound **13** was obtained as a white solid in 100% yield. ¹H NMR (300 MHz, CD₃OD) δ 6.48 (s, 1H), 2.49 (t, J = 7.6 Hz, 2H), 1.65–1.51 (m, 2H), 1.45–1.30 (m, 2H), 0.95 (t, J = 7.3 Hz, 3H) ppm. ¹³C NMR (75 MHz, CD₃OD) δ 147.0, 127.8, 108.1, 30.0, 23.7, 21.7, 12.6 ppm. HRMS (ESI) calcd for C₇H₁₃N₃ [M + H]⁺ 140.1182, found 140.1179.

4-(5-Aminopentyl)-1*H*-imidazol-2-amine (**10**).

Using the general procedure for Boc deprotection, compound **10** was obtained as an orange solid in 94% yield. ¹H NMR (600 MHz, CD₃OD) δ 6.46 (s, 1H), 2.87 (t, J = 7.6 Hz, 2H), 2.47 (t, J = 7.6 Hz, 2H), 1.68–1.55 (m, 4H), 1.39 (p, J = 7.8 Hz, 2H) ppm. ¹³C NMR (150 MHz, CD₃OD) δ 148.5, 128.7, 109.8, 40.6, 28.7, 28.2, 26.7, 25.2 ppm. HRMS (ESI) calcd for C₈H₁₆N₄ [M + H]⁺ 169.1448, found 169.1446.

5-(3-Amino-1-(2-amino-1*H*-imidazol-4-yl)propyl)-1*H*-imidazol-2-amine (**1**).

Using the general procedure for oxidative heterodimerization, compound **1** was obtained as an orange solid in 5% yield. ¹H NMR (300 MHz, D₂O) δ 6.75 (s, 2H), 4.10 (t, J = 7.7 Hz, 1H), 3.01 (t, J = 8.1 Hz, 2H), 2.27 (q, J = 7.9 Hz, 2H) ppm. ¹³C NMR (125 MHz, D₂O) δ 147.6, 125.0, 110.9, 37.2, 30.2, 28.8 ppm. HRMS (ESI) calcd for C₉H₁₅N₇ [M + H]⁺ 222.1462, found 222.1459.

5-(4-Amino-1-(2-amino-1H-imidazol-4-yl)butyl)-1H-imidazol-2-amine (2).

Using the general procedure for oxidative heterodimerization, compound **2** was obtained as an orange solid in 5% yield. ¹H NMR (400 MHz, D₂O) δ 6.39 (s, 2H), 3.57 (t, *J* = 7.7 Hz, 1H), 2.60 (t, *J* = 7.2 Hz, 2H), 1.79 (q, *J* = 8.0 Hz, 2H), 1.38 (p, *J* = 7.4 Hz, 2H) ppm. ¹³C NMR (125 MHz, D₂O) δ 147.4, 126.0, 110.6, 39.0, 32.1, 28.1, 24.5 ppm. HRMS (ESI) calcd for C₁₀H₁₇N₇ [M + H]⁺ 236.1618, found 236.1616.

5-(5-Amino-1-(2-amino-1H-imidazol-4-yl)pentyl)-1H-imidazol-2-amine (16).

Using the general procedure for oxidative heterodimerization, compound **16** was obtained as an orange solid in 5% yield. ¹H NMR (300 MHz, D₂O) δ 6.46 (s, 2H), 3.64 (t, *J* = 7.8 Hz, 1H), 2.92 (t, *J* = 7.4 Hz, 2H), 1.85 (q, *J* = 7.7 Hz, 2H), 1.70–1.56 (m, 2H), 1.39–1.24 (m, 2H) ppm. ¹³C NMR (175 MHz, D₂O) δ 147.1, 126.3, 110.2, 39.2, 33.1, 30.4, 26.8, 23.2 ppm. HRMS (ESI) calcd for C₁₁H₁₉N₇ [M + H]⁺ 250.1775, found 250.1774.

5-(1-(2-Amino-1H-imidazol-4-yl)butyl)-1H-imidazol-2-amine (17).

Using the general procedure for oxidative heterodimerization, compound **17** was obtained as a brown solid in 7% yield. ¹H NMR (400 MHz, CD₃OD) δ 6.70 (s, 2H), 3.91 (t, *J* = 7.6 Hz, 1H), 1.91 (q, *J* = 7.7 Hz, 2H), 1.41–1.31 (m, 2H), 0.98 (t, *J* = 7.3 Hz, 3H) ppm. ¹³C NMR (125 MHz, CD₃OD) δ 149.1, 128.3, 111.4, 35.0, 33.8, 21.3, 13.9 ppm. HRMS (ESI) calcd for C₁₀H₁₆N₆ [M + H]⁺ 221.1509, found 221.1506.

5-(1-(2-Amino-1H-imidazol-4-yl)tridecyl)-1H-imidazol-2-amine (18).

Using the general procedure for oxidative heterodimerization, compound **18** was obtained as a brown solid in 10% yield. ¹H NMR (400 MHz, CD₃OD) δ 6.70 (s, 2H), 3.89 (t, *J* = 7.7 Hz, 1H), 1.93 (q, *J* = 7.4 Hz, 2H), 1.34–1.27 (m, 20H), 0.89 (t, *J* = 6.9 Hz, 3H) ppm. ¹³C NMR (125 MHz, CD₃OD) δ 149.0, 128.3, 111.4, 34.1, 33.1, 33.0, 30.8, 30.8, 30.7, 30.7, 30.6, 30.5, 30.3, 28.2, 23.7, 14.5 ppm. HRMS (ESI) calcd for C₁₉H₃₄N₆ [M + H]⁺ 347.2918, found 347.2915.

5-(1-(2-Amino-1H-imidazol-4-yl)pentadecyl)-1H-imidazol-2-amine (19).

Using the general procedure for oxidative heterodimerization, compound **19** was obtained as a brown solid in 6% yield. ¹H NMR (700 MHz, CD₃OD) δ 6.61 (s, 2H), 3.79 (t, *J* = 7.7 Hz, 1H), 1.87–1.78 (m, 2H), 1.53–0.93 (m, 24H), 0.82 (t, *J* = 7.0 Hz, 3H) ppm. ¹³C NMR (175 MHz, CD₃OD) δ 149.7, 128.9, 111.8, 40.0, 40.0, 34.5, 33.6, 33.4, 31.3, 31.2, 31.2, 31.2, 31.1, 31.0, 30.7, 28.7, 24.2, 14.9 ppm. HRMS (ESI) calcd for C₂₁H₃₈N₆ [M + H]⁺ 375.3231, found 375.3234.

8-Ethyl-4-propyl-3H-cyclohepta[1,2-*d*:4,5-*d'*]-diimidazole-2,6-diamine (20).

Using the general procedure for oxidative heterodimerization, compound **20** was obtained as a brown solid in 4% yield. ¹H NMR (300 MHz, CD₃OD) δ 7.87 (s, 1H), 3.41–3.33 (m, 2H), 3.19 (q, *J* = 7.5 Hz, 2H), 1.82–1.69 (m, 2H), 1.38 (t, *J* = 7.5 Hz, 3H), 1.11 (t, *J* = 7.3 Hz, 3H) ppm. ¹³C NMR (175 MHz, CD₃OD) δ 162.8, 161.6, 150.1, 148.3, 147.1, 147.0, 139.5, 130.0, 121.8, 32.6, 29.0, 22.5, 13.9, 13.2 ppm. HRMS (ESI) calcd for C₁₄H₁₈N₆ [M + H]⁺ 271.1666, found 271.1663.

4-Dodecyl-8-undecyl-3*H*-cyclohepta[1,2-*d*:4,5-*d'*]-diimidazole-2,6-diamine (**21**).

Using the general procedure for oxidative heterodimerization, compound **21** was obtained as a brown solid in 4% yield. ¹H NMR (300 MHz, CD₃OD) δ 7.84 (s, 1H), 3.38 (t, J = 8.2 Hz, 2H), 3.13 (t, J = 7.8 Hz, 2H), 1.77–1.66 (m, 4H), 1.26–1.16 (m, 34H), 0.87 (t, J = 5.9 Hz, 6H) ppm. ¹³C NMR (175 MHz, CD₃OD) 180.2, 173.3, 171.2, 162.2, 160.9, 149.2, 141.1, 137.5, 130.6, 125.7, 122.5, 75.2, 50.8, 48.1, 35.9, 31.8, 31.7, 30.9, 30.2, 29.8, 29.6, 29.5, 29.4, 29.4, 29.4, 29.3, 29.3, 29.3, 29.2, 29.1, 22.3, 13.0 ppm. HRMS (ESI) calcd for C₃₂H₅₄N₆ [M + H]⁺ 523.4483, found 523.4486.

Bacterial Strains and Antimicrobial Agents.

Seven strains of *Pseudomonas aeruginosa* and one strain of *Klebsiella pneumoniae* were the bacterial strains used in this study. Cystic fibrosis clinical isolates (three nonmucoid strains CEC 45, 57, and 102 and three mucoid strains CEC 70, 103, and 111) were obtained from Robert K. Ernst, in the Department of Microbial Pathogenesis at University of Maryland—Baltimore. All strains were maintained and subcultured in cation-adjusted Mueller-Hinton broth (CAMHB) in liquid culture or on lysogeny broth (LB) plates until they were utilized in various assays outlined below. Antibiotics were purchased from Sigma-Aldrich or TCI America. All assays were run in duplicate and repeated at least two separate times. All compounds were dissolved as their HCl salts in molecular-biology-grade DMSO as a 10 or 100 mM stock solution and stored at –20 °C.

Bacterial Membrane Permeabilization Assay.

The BacLight assay (Invitrogen) was used to assess the membrane permeability. PAO1 was grown overnight in CAMHB at 37 °C with shaking. The culture was diluted 1:40 in CAMHB and grown to an optical density at 600 nm (OD₆₀₀) of ~1.0 (~4 h of growth). The cultures were centrifuged at 10 000*g* for 15 min, and then the cell pellet was washed once with sterile water, resuspended at 2 times the original volume, and divided into aliquots before adjuvants were added. Suspensions were incubated at 37 °C with shaking for 1 h and then centrifuged at 10 000*g* for 10 min, washed once with sterile water, and resuspended in water. A 1:1 mixture of SYTO-9/propidium iodide was added to the suspension (3 μ L/mL) and mixed well. The suspension (100 μ L) was added to each well of a 96-well plate, and the plates were incubated in the dark for 15 min at room temperature. Green fluorescence (SYTO-9) was read at 530 nm, and red fluorescence (propidium iodide) was read at 645 nm (excitation wavelength, 485 nm). The ratio of green to red fluorescence was expressed as a percentage of the control.

Bacterial Membrane Permeabilization Assay with Divalent Cation Metals.

The procedure for the bacterial membrane permeabilization assay was followed, with the addition of using 200 μ M FeSO₄·7H₂O or MnCl₂ to spike a suspension of bacterial solution containing the test compound before incubating at 37 °C with shaking for 1 h.

Broth Microdilution Method for the Determination of the Minimum Inhibitory Concentrations.

Day cultures (6 h) of each bacterial strain in cation-adjusted Mueller Hinton II broth (CAMHB, Fisher Scientific U.S.) were subcultured to 5×10^5 CFU/mL in CAMHB. Aliquots (1 mL) were placed in culture tubes, and compound was added from a 100 or 10 mM stock sample in DMSO such that the compound concentration equaled the highest concentration tested (200 μ M). Aliquots of samples (200 μ L) were then into the first wells of a 96-well plate, with all remaining wells being filled with 100 μ L of the initial bacterial subculture. Row one wells were mixed five times before 100 μ L was transferred to row two. Row two was then mixed five times, and 100 μ L was transferred to row three. This process was repeated until the final row had been mixed, which served to serially dilute the compound. Plates were then covered with GLAD Press'n Seal and incubated under stationary conditions at 37 °C for 16 h. MIC values were then recorded as the lowest concentration at which no bacterial growth was observed.

Broth Microdilution Method for Antibiotic Potentiation.

Day cultures (6 h) of bacteria in CAMHB were subcultured to 5×10^5 CFU/mL in CAMHB. Aliquots (4 mL) were placed in culture tubes and dosed with compound from 100 or 10 mM stock samples to give the desired concentration of the compound to be tested against the particular bacterial strain; this ensured nontoxic DMSO concentrations of 0.3% in each well. One milliliter of the resulting solution was placed in a separate culture and dosed with antibiotic at the highest concentration to be tested. Bacteria treated with antibiotic alone served as the control. Row 1 of a 96-well plate was filled with 200 μ L of the antibiotic/2-AI solution, and rows 2–12 were filled with 100 μ L each of the remaining 4 mL of bacterial subculture containing adjuvant at the desired concentration except for the control lane, which contained only bacterial subculture. Row one was then mixed five times, and 100 μ L was transferred to row two, which was then mixed five times before being transferred to row three. This process was repeated until all rows had been mixed, except for row 12, which would contain only compound to serve as a control. The 96-well plate was then covered in GLAD Press'n Seal and incubated under stationary conditions at 37 °C for 16 h. MIC values were determined to be the lowest concentration at which no bacterial growth was observed, and fold reductions were determined by comparison to the control lane.

Broth Microdilution Method for Antibiotic Potentiation with Detergent.

Day cultures (6 h) of bacteria in CAMHB were subcultured to 5×10^5 CFU/mL in CAMHB, and then Tween 80 Surfact-Amps detergent solution (Fisher Scientific, U.S.) was added to make a 0.01% bacterial solution. Then the procedure for the broth microdilution method for antibiotic potentiation was followed, starting with making 4 mL aliquots from Tween 80 bacterial solution.

Dose–Response of the Adjuvant Assay.

The procedure for the broth microdilution method for antibiotic potentiation was followed using 4 mL aliquots of adjuvant at various concentrations: 0, 15, 30, 35, 40, 50, and 60 μ M.

EDTA Assay.

The procedure for the broth microdilution method for antibiotic potentiation was followed, with the addition of doping the 4 mL aliquots containing adjuvant with either a 25, 50, 100, or 200 μ M prepared EDTA stock. Bacterial solutions lacking adjuvant were used as controls to examine the effect of 200 μ M EDTA on *P. aeruginosa* growth. Row one of a 96-well plate was filled with 200 μ L of the antibiotic/adjuvant/EDTA solution, and rows two through eight were filled with 100 μ L each of the remaining bacterial subculture dosed with adjuvant/EDTA. The serial dilution of rows followed as previously stated. Plates were covered with GLAD Press'n Seal and incubated under stationary conditions at 37 °C. After 16 h, MIC values were determined as the lowest concentration at which no bacterial growth was observed, and fold reductions were determined by comparison to control lanes.

Galleria mellonella Assay.

Galleria mellonella larvae (Speedy Worm, Alexandria, MN) were used within 10 days of shipment from the vendor. After the reception of worms, larvae were kept in the dark at room temperature for at least 24 h before infection. Larvae weighing between 200 and 300 mg were used in the survival assay. Using a 10 μ L glass syringe (Hamilton, Reno, NV) fitted with a 30 G needle (Exel International, St. Petersburg, FL), a 5 μ L solution of the desired compound(s) and concentration(s) was injected into the last left proleg. Azithromycin was dosed at 1 mg/kg dissolved in DI water, 2-AIs was dosed at 50 mg/kg dissolved in DMSO, meropenem was dosed at 30 mg/kg dissolved in DMSO, and DMSO was injected for "bacteria only" as a control. For bacterial injections, 50 μ L from an overnight culture of *P. aeruginosa* (PAO1) in Miller LB broth (Fisher Scientific U.S.) was subcultured into 5 mL of Miller LB broth and incubated for an additional 3 h before use. Then, 2.5 h after the first injection, a second 5 μ L injection containing 10⁵ CFU of PAO1 in phosphate-buffered solution (Fisher Scientific U.S.) was injected into the second-to-last left proleg. Injected worms were left at room temperature in the dark while being assessed at 24 h intervals over 6 days. Larvae were considered to be dead if they did not respond to physical stimuli. The experiment was repeated three times using 10 larvae per experimental group. No ethics approval was needed for *G. mellonella*.

Hemolysis Assay.

Hemolysis assays were performed on mechanically defibrinated sheep blood (Hemostat Laboratories, DSB100). Defibrinated blood (1.5 mL) was placed into a microcentrifuge tube and centrifuged for 10 min at 10 000 rpm. The supernatant was then removed, and then the cells were resuspended in 1 mL of phosphate-buffered saline (PBS). The suspension was centrifuged, the supernatant was removed, and cells were resuspended two additional times. The final cell suspension was then diluted 10-fold. A test compound was added to aliquots of the 10-fold suspension dilution of blood. PBS was used as a negative control and a zero hemolysis marker. Triton X (a 1% sample) was used as a positive control, serving as the 100% lysis marker. Samples were then placed in an incubator at 37 °C while being shaken at 200 rpm for 1 h. After 1 h, the samples were centrifuged for 10 min at 10 000 rpm. The resulting supernatant was diluted by a factor of 40 in distilled water. The absorbance of the supernatant was then measured with a UV spectrometer at a 540 nm wavelength.

Minimal Salts Assay.

The procedure for the broth microdilution method for antibiotic potentiation was followed, with the exception of culturing *P. aeruginosa* in LB broth for 6 h and then subculturing in minimal salts media (M9) instead of CAMHB.

Procedure to Determine the Mitigating Effects of Divalent Cation Metals on *P. aeruginosa*.

The procedure for the broth microdilution method for antibiotic potentiation was followed, with the addition of doping the 4 mL aliquots containing adjuvant with either 25, 50, 100, or 200 μ M divalent cation metal ($\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, ZnCl_2 , CaCl_2 , CuCl_2 , MgCl_2 , or MnCl_2). Controls in which no adjuvant was added were used to examine the effects of 200 μ M metal on *P. aeruginosa* growth.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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ABBREVIATIONS

2-ABI	2-aminobenzimidazole
2-AI	2-aminoimidazole
azm	azithromycin
CF	cystic fibrosis
CFTR	cystic fibrosis transmembrane conductance regulator
CLSI	Clinical and Laboratory Standards Institute
MDR	multidrug resistance
MIC	minimum inhibitory concentration
PI	propidium iodide
r.t.	room temperature
SAR	structure–activity relationship

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Figure 1.
Initial library bis-2-AIs screened for the enhancement of azithromycin activity against a highly resistant strain of *P. aeruginosa*.

Figure 2.
Percent survival of *G. mellonella* after an antibiotic-adjuvant, antibiotic, or DMSO (control, i.e., bacteria only) dosage followed by inoculation with PAO1.

Scheme 1.
Synthesis of the Core 2-AI Scaffold

$\text{R} = -(\text{CH}_2)_2\text{NH}_2$ (**6**)

$\text{R} = -(\text{CH}_2)_3\text{NH}_2$ (**7**)

$\text{R} = -(\text{CH}_2)_4\text{NH}_2$ (**10**)

$\text{R} = -(\text{CH}_2)_2\text{CH}_3$ (**13**)

$\text{R} = -(\text{CH}_2)_{11}\text{CH}_3$ (**14**)

$\text{R} = -(\text{CH}_2)_{13}\text{CH}_3$ (**15**)

$\text{R}' = -(\text{CH}_2)_2\text{NH}_2$ (**1**), 5%

$\text{R}' = -(\text{CH}_2)_3\text{NH}_2$ (**2**), 5%

$\text{R}' = -(\text{CH}_2)_4\text{NH}_2$ (**16**), 5%

$\text{R}' = -(\text{CH}_2)_2\text{CH}_3$ (**17**), 7%

$\text{R}' = -(\text{CH}_2)_{11}\text{CH}_3$ (**18**), 10%

$\text{R}' = -(\text{CH}_2)_{13}\text{CH}_3$ (**19**), 6%

$n_1 = 1, n_2 = 2$ (**20**), 4%

$n_1 = 10, n_2 = 11$ (**21**), 4%

Scheme 2.

Synthesis of Bis-2-AI Analogues

Table 1.

Azithromycin Potentiation Data for Bis-2-AI Derivatives against PAO1

compound	compound MIC (μ M)	compound concentration tested (μ M)	Azithromycin MIC (μ g/mL) ^a	fold reduction
1	>200	60	32	8
2	200	60	0.25	1024
3	>200	60	128	2
16	>200	60	16	16
17	>200	60	128	2
18	>200	30	256	0
19	100	30	128	2
20	>200	60	128	2
21	>200	30	256	0

^a Azithromycin MIC alone is 256 μ g/mL.

Table 2.

Dose Response of Compound 2 against PAO1

compound	compound concentration tested (μ M)	azithromycin MIC (μ g/mL)	fold reduction
2	0	256	0
	15	64	4
	30	16	16
	35	8	32
	40	1	256
	50	0.25	1024
	60	0.25	1024

Table 3.

Metal Doping Effect on the Ability of Compound 2 to Potentiate Azithromycin Activity against PAO1

conc of metal tested (μM) ^a	azithromycin MIC ($\mu\text{g/mL}$) ^{b,c} + 2 ^d					
	FeSO ₄ ·7H ₂ O	ZnCl ₂	CaCl ₂	CuCl ₂	MgCl ₂	MnCl ₂
0	0.5	0.25	0.12	0.25	0.5	0.25
50	16	0.25	0.25	0.25	0.5	2
100	64	0.25	0.25	0.25	1	4
200	128	0.5	2	0.5	1	16

^aEach divalent metal exhibited MIC > 200 μM when dosed alone.^bAzithromycin's MIC was 256 $\mu\text{g/mL}$ against untreated PAO1.^cAzithromycin's MIC was 128 $\mu\text{g/mL}$ when combined with each divalent metal at 200 μM .^dCompound 2 was tested at 60 μM .

Table 4.

Additional Antibiotic Potentiation of Bis-2-AI 2 against PAO1

antibiotic	azithromycin MIC ($\mu\text{g/mL}$)	azithromycin MIC ($\mu\text{g/mL}$) + 2 ^a	fold reduction
clarithromycin	256	2	128
doripenem	2	0.06	32
rifampin	32	0.5	64
colistin	2	1	2

^aCompound 2 was tested at 60 μM .

Table 5.

Azithromycin Potentiation Data for Bis-2-AI 2 against Cystic Fibrosis Strains

cell type	CF strains ^a	compound 2 MIC (μ M)	azithromycin MIC ^b (μ g/mL)	azithromycin MIC (μ g/mL) + 2	fold reduction
nonmucoid	CEC 45	>200	256	0.5	512
	CEC 57	200	256	8	32
	CEC 102	>200	256	2	128
mucoid	CEC 70	200	256	0.06	4267
	CEC 103	>200	128	4	32
	CEC 111	50	128	8 ^c	16

^aCystic fibrosis strains (three nonmucoid strains CEC 45, 57, and 102 and three mucoid strains CEC 70, 103, and 111) were obtained from Robert K. Ernst in the Department of Microbial Pathogenesis at University of Maryland—Baltimore.

^bAll MIC values are in μ g/mL, and compound 2 tested at 60 μ M for all strains except for CEC 111.

^cCompound 2 tested at 15 μ M.