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Antibacterial Diamines Targeting Bacterial

Membranes

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ABSTRACT

Antibiotic resistance is a growing threat to human health exacerbated by a lack of new antibiotics. We now describe a series of substituted diamines that produce rapid bactericidal activity against both Gram-positive and Gram-negative bacteria, including methicillin-resistant *Staphylococcus aureus* and stationary-phase bacteria. These compounds reduce biofilm formation and promote biofilm dispersal in *Pseudomonas aeruginosa*. The most potent analogue, **3** (1,13-bis{[(2,2-diphenyl)-1-ethyl]thioureido}-4,10-diazatridecane), primarily acts by depolarization and permeabilization of the bacterial outer membrane. Transmission electron microscopy confirmed that **3** disrupts membrane integrity rapidly. Compound **3** is also synergistic with kanamycin, demonstrated by the checkerboard method and by time-kill kinetic experiments. In human cell toxicity assays, **3** showed limited adverse effects against the HEK293T human kidney embryonic cells and A549 human adenocarcinoma cells. In addition, **3** produced no adverse effects on *Caenorhabditis elegans* development, survival and reproduction. Collectively, diamines related to **3** represent a new class of broad-spectrum antibacterials against drug-resistant pathogens.

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Introduction

Infections caused by drug-resistant bacteria have become an increasing threat to human health. Despite the rapidly increasing incidence of antibacterial resistance, few new antibiotics are currently being developed.[1] Thus, there is an urgent need to identify new antibiotics from distinct chemical classes and with novel mechanisms of action. Unlike conventional antibiotics, cationic antimicrobial peptides (AMPs) are known to act primarily by disrupting the bacterial cell membrane. Moreover, this charge-charge interaction between AMPs and bacterial cell membrane does not target intracellular bacterial organelles. Thus, bacteria find it difficult to develop resistance against AMPs.[2] However, AMPs are mainly limited by their potential for toxicity, high susceptibility to proteolytic degradation, and the high cost of manufacture. In the recent past, several classes of cationic small molecules that mimic the AMPs have shown some promise as potential antibiotics, and some of them are undergoing clinical trials.[3-7]

By virtue of the cationic groups in their structure, natural polyamines exhibit a broad range of biological functions that promote bacterial fitness and pathogenesis.[8] Recent studies have shown that natural polyamines and their analogues can impair membrane integrity and biofilm formation[9-11] without affecting planktonic bacterial growth,[12] and also increase antibiotic susceptibility.[13, 14] Synthetic polyamine-containing sterol analogues related to squalamine also exhibit antimicrobial activities.[15-17] These data suggest that there is considerable potential for the use of synthetic diamines as potential antibacterial drug candidates.

Herein we report the design and development of synthetic diamines **1-16** (Figure 1); compounds **1-12** are bactericidal against *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Escherichia coli*, and methicillin-resistant *Staphylococcus aureus* (MRSA), while **13-16** were designed to evaluate the requirement for the central charged nitrogens. Importantly, these

compounds also strongly inhibit biofilm formation and promote biofilm dispersal in the gram-negative pathogen *P. aeruginosa*. The most effective of these analogues, compound **3**, was selected for use as a chemical tool to elucidate the exact mechanism of the bactericidal activity for these agents, which until now was unclear. We hypothesize that positively charged diamines such as **1-12** can interact with the negatively charged bacterial cell membrane. Therefore, we employed multiple methods to elucidate the effects of synthetic diamines on membrane homeostasis. We also determined the effect of **3** in combination with selected commercial antibiotics to determine whether they act synergistically. Finally, we determined the effect of **3** against non-dividing bacterial cells and evaluated its protease tolerance and toxicity in human cells and *Caenorhabditis elegans*. Findings from this study provide evidence that diamines are membrane-targeting inhibitors that are active against drug-resistant pathogens.

Chemistry

The (bis)ureido- and (bis)thioureido diamines **1-12** described in this manuscript were synthesized according to our recently published pathway,[18] as shown in Scheme 1. The synthesis of compound **3** is described below, and all other compounds were synthesized in exactly the same manner. 1,5-Diaminopentane **17** was (bis)cyanoethylated (acrylonitrile, ethanol, reflux) and the central nitrogens were protected [(Boc)$_2$O, NaHCO$_3$, CHCl$_3$/H$_2$O] to afford the previously described (bis)-N-Boc protected intermediate **18**.[19, 20] Compound **18** was reduced in the presence of Raney nickel (H$_2$, 50 psi, NH$_4$OH) to form the (bis)-N-Boc protected diamine **19**. Intermediate **19** was then reacted with the appropriate isocyanate or isothiocyanate (in this case 2,2-diphenylethyl isothiocyanate **20**) to yield the protected precursor **21**. Removal of the N-Boc protecting groups with mild acid (HCl in EtOAc) then afforded the desired target compound **3**.

Compounds **13**-**16**, which lack the two internal secondary nitrogens found in **1**-**12**, were synthesized as described in Scheme 2. Briefly, 1,11-diaminoundecane **22** was allowed to react with 2 equivalents of 3,3-diphenylpropyl isocyanate **23** to afford the corresponding target compound **13**. Likewise, 1,12-diaminododecane **24** was coupled to the appropriate isocyanate or isothiocyanate **25-27** to afford the corresponding analogues **14-16**.

Results

Antibacterial screen. In order to determine whether synthetic diamines **1**-**16** possessed an acceptable minimum inhibitory concentration (MIC) in multiple organisms, the MIC_{99} of compounds **1**-**16** was determined against a Gram-positive organism (*Staphylococcus aureus* strain RN4220) and 2 Gram-negative organisms (*Pseudomonas aeruginosa* strain PA01 and *Escherichia coli* strain ANS1). The MIC_{99} assay for the antibacterial activity of each test compound was performed by the micro-broth dilution method of the Clinical and Laboratory Standards Institute of America. MIC_{99} was defined as the lowest compound concentration at which bacterial growth was completely inhibited after overnight incubation in a 96-well plate (Nunc) at 37°C without shaking. The results of these studies are summarized in Table 1. Commercially available antibiotics from 4 different classes were used as positive controls: the penicillin derivative ampicillin (AMC), the aminoglycoside kanamycin (KAN), the fluoroquinoline norfloxacin (NOR) and the polymixin antibiotic colistin. The natural polyamines spermidine and spermine were also assayed as negative control compounds. Synthetic diamines **1-4** and **7-12** produced significant antibacterial activity, with MIC_{99} values between 2 and 32 μg/mL (with one exception: compound **1** had an MIC_{99} value of 256 μg/mL against *P. aeruginosa*). The most effective of these analogues, **3**, exhibited MIC_{99} values of 2.0, 8.1 and 2.2

μg/mL against *S. aureus* RN4220, *P. aeruginosa* PA01 and *E. coli* ANS1, respectively. Compounds **13-16** were synthesized to determine whether the presence of the two central nitrogens was required for activity. Importantly, compounds **13-16** produced no detectable inhibitory effect against any of the 3 tested strains. A direct comparison could be made between active compounds **10-12** and inactive (control) analogues **14-16**, since each of these compounds possessed a chain length of 12 atoms between the terminal substituted ureas or thioureas. Substitution of carbon for the internal nitrogens in **10-12** produced a complete abolition of antibacterial activity. Based on the fact that simple polyamine analogues such as spermidine and spermine do not produce antibacterial effects, it would appear that the presence of both the central charged nitrogens and the terminal diaryl moieties are required for activity. It may be that the aromatic ring structures produce additional affinity for the bacterial membrane due to a hydrophobic effect. However, this effect in itself in not sufficient to produce the observed antibacterial activity, since **13-16** are inactive. Interestingly, it has been shown that the natural polyamines spermidine and spermine, as well as simple diamines like putrescine and cadaverine, have no antibacterial effects, no effect on outer membrane permeability, and did not cause membrane rupture.[13] These amine derivatives have no inherent antibacterial activity in pseudomonas, and are in fact biosynthesized and imported by numerous bacteria, including *Pseudomonas aeruginosa*,[21] *Staphylococcus aureus*[22] and *Escherichia coli*.[22] Although diamines such as those mentioned above have no inherent antibacterial activity, addition of exogenous spermidine (1.0 mM) or other polyamines to the pseudomonas growth medium increased the MIC values of multiple antibiotics, including polycationic antibiotics, aminoglycosides, quinolones, and fluorescent dyes.[23] In a subsequent study by the same group, spermine was shown to decrease the MIC values for chloramphenicol and b-lactam antibiotics.[14] Taken

together, these data support the contention that both the central charged nitrogens and the terminal diaryl moieties in **1-12** are required for the observed antibacterial activity.

A clear structure/activity trend was also observed by comparing the MIC_{99} values of compounds **1-12**. Compounds with (bis)aryl substitutions on the terminal alkyl group were significantly more potent than those with monoaryl substituents, as reflected in MIC_{99} values. Also, the (bis)arylthioureido analogues were slightly more effective than the corresponding (bis)arylureido analogues, although this difference may not be indicative of a trend. Also, as outlined above, the central charged nitrogen species in **1-12** are required for activity. Because compound **3** was very effective in all three organisms, it was chosen for the experiments described below.

*Antibacterial activity of compound **3** against methacillin-resistant Staphylococcus aureus.* To further probe the activity of antimicrobial diamines **1-12**, compound **3** was evaluated for the ability to kill methacillin-resistant *Staphylococcus aureus* (MRSA) in vitro. Five MRSA isolates from the Medical University of South Carolina collection were selected for antibacterial activity testing. These isolates represented the most commonly found SPA types in the MUSC collection. Strain 10082 B is SPA type 1, 10076 B is SPA type 2, 30253 CA is SPA type 7, 20225 B is SPA type 15, and 20467 BA is SPA type 59. Compound **3** exhibited potent anti-MRSA activity, exhibiting MIC_{99} values of 1-2 μg/mL against each isolate (Table 2). These MIC_{99} values are comparable to the MIC value of **3** against *S. aureus* RN4220 (2 μg/mL, see Table 1).

*Effect of compound **3** on biofilm formation in Pseudomonas aeruginosa.* As shown in Figure 2, compound **3** inhibited *P. aeruginosa* biofilm formation at 64 μg/mL, which was comparable to the commercial antibiotics tetracycline (TET) and NOR, whereas KAN displayed no inhibitory effect below 1024 μg/mL (Figure 2A). Interestingly, **3** had a significantly stronger effect on the

dispersal of formed biofilm (Figure 2B) when compared to TET, NOR and KAN. The term drug tolerance refers to the non-inherited drug resistance that develops against antibiotics in non-dividing cells such as bacteria in the stationary phase.[24] Because we suspected that diamines target the bacterial membrane (see below), we hypothesized that they would be bactericidal against non-dividing cells. Thus the effect of compound **3** on drug tolerant cells was evaluated using bacteria in stationary phase. Actively dividing cells in exponential phase were used for the purpose of comparison. An in vitro time-kill assay for **3** against stationary phase *S. aureus* and *P. aeruginosa* revealed the remarkable and rapid bactericidal activity against both multiplying bacteria and non-dividing stationary cells (Figure 3). By contrast, the traditional antibiotics AMC, KAN and NOR displayed strong bactericidal activity against dividing bacteria, but did not kill stationary phase bacteria efficiently. When tested at bactericidal concentrations (4X MIC_{99}), AMC, KAN, NOR and compound **3** showed similar bactericidal activity against log phase *S. aureus* cells (Figure 3A). However, stationary-phase *S. aureus* were resistant to AMC, KAN and NOR, while remaining susceptible to **3** (Figure 3B). Following 4-hour treatment, NOR reduced the viable cell numbers only by 2 logs while **3** eliminated all viable cells. Log phase *P. aeruginosa* responded weakly to AMC and KAN, while NOR and **3** both produced a 4 log reduction in viable bacteria (Figure 3C). *P. aeruginosa* in stationary phase was completely resistant to AMC and KAN, and displayed increased resistance to NOR compared to log phase cells. However, compound **3** elicited potent bactericidal activity against stationary phase *P. aeruginosa* (Figure 3D), and eliminated all bacteria after 4 hours. These data demonstrate that the membrane-targeting diamine **3** can circumvent drug tolerance in non-dividing cells, since both dividing log phase and non-dividing stationary phase bacteria require intact membranes to survive.

Antibacterial activity in plasma. One of the major disadvantages of AMPs is their potential for proteolytic inactivation. This is reflected in the subsequent loss of antibacterial activity of AMPs in the presence of plasma.[25] To determine whether antibacterial diamines are resistant to hydrolysis by proteases, the MIC_{99} value of **3** in the presence of plasma was measured. A stock solution was prepared containing 10% fetal bovine serum (FBS), and the MIC_{99} assay was performed by the micro-broth dilution method as described above. Following 12 hour pre-incubation in the presence of plasma, **3** displayed no loss of activity when cultured in the presence of 10% FBS (data not shown).

Hemolytic activity and cytotoxicity. The toxicity of compound **3** in mammalian cells was measured by its ability to lyse human red blood cells using the method of Kustanovich,[26] and the results were represented as the HC_{50} value (the concentration at which 50% of the red blood cells are lysed). The HC_{50} of compound **3** was 64 µg/mL, indicating that the compound produced selective toxicity towards bacterial cells. The cytotoxicity of compound **3** was also monitored in two additional mammalian cell lines, the HEK293T human kidney embryonic cell line and the A549 human alveolar basal epithelial adenocarcinoma cell line. The results of these studies are shown in Table 3. The selectivity index (SI) in each cell line was the ratio of the CC_{50} value of cytotoxicity against a given human cell line divided by the MIC_{99} value for a given bacterial strain. Compound **3** possessed a favorable SI (defined as an $SI \geq 10$) between 16 and 64 against *S. aureus*, *E. coli* and MRSA clinical isolates. The SI against *P. aeruginosa* was lower (either 4 or 8), suggesting that additional structure optimization is required to identify a compound that kills *P. aeruginos*a with an acceptable SI.

Toxicity against Caenorhabditis elegans. C. elegans has been successfully used to study acute bacterial infections,[27-29] and has also been employed to assess toxicity.[30] For our purposes, we

incubated *C. elegans* with high concentrations of **3**, gentamicin and 5-fluorodideoxyuridine (5FU). Gentamicin and 5FU are both toxic towards eukaryotic cells at high concentrations and were used as controls throughout the assay.[31, 32] Prior to this study, we evaluated the effect of lysed *E. coli* as a food source for nematodes because **3** has demonstrated the ability to lyse bacterial membranes. *C. elegans* incubated with mechanically-lysed *E. coli* displayed no alterations in development and procreation (data not shown). *C. elegans* eggs, L1 and L4 development stages were incubated with 64 μg/mL of **3** for 96 h, which provided enough time to assess the complete life cycle. We observed no changes in *C. elegans* egg development, and larvae that hatched produced viable eggs and displayed no deleterious genotypic effects. Juvenile and adult nematodes were also not affected as evidenced by 100% survival, no changes in locomotion, and maintenance of egg production. High concentrations of gentamicin or 5FU were toxic towards *C. elegans*, specifically affecting the viability of eggs produced and killing juvenile nematodes. Collectively, the assessment of the toxicity of **3** in *C. elegans* suggests that the compound does not produce overt toxicity *in vivo* and should be further evaluated in a higher organism. These data also indicate that the toxicity of **3** is low, an observation that is in agreement with our results from toxicity testing in human cells.

Mechanism of action. The mechanism of action of compound **3** was investigated using various spectroscopic methods that revealed that the diamines primarily acted by targeting the bacterial cell membrane. As described below, our results show that the diamines exhibit rapid and potent bactericidal activity. This fast killing activity was indicative of membrane targeting activities, which could be mediated by an electrostatic interaction between the positively charged central nitrogens in **3** and its analogues, and the negatively charged bacterial membrane. This hypothesis is consistent with the fact that positively charged AMPs can disrupt bacterial membrane integrity

and depolarize bacterial membranes.[33] To test this hypothesis, we first measured the effect of **3** on bacterial membrane potential using a membrane-potential sensitive fluorescent probe ($DiSC_3$-5). In buffer-treated control cells, the fluorescent signal of the probe was quenched by cell membranes with intact membrane potential (Figure 4A and B). The addition of compound **3** at 4X MIC_{99} (8 µg/mL) and 1X MIC_{99} (2 µg/mL) concentrations against *S. aureus* caused a rapid increase in the probe fluorescence intensity (Figure 4A), indicating the membrane potential was dissipated by the diamine. Treatment of *P. aeruginosa* by compound **3** led to a similar abrupt increase in $DiSC_3$-5 fluorescence (Figure 4B), demonstrating the cell membrane of both Gram-positive and Gram-negative bacteria can be depolarized. There is now increasing evidence that cationic antimicrobial agents can depolarize the cytoplasmic membrane. However, it is unclear if depolarization is a consequence of the dying bacteria or is truly tied to the mechanism. To this end, we performed simultaneous testing of bactericidal kinetics and depolarization (Figure 5). Following a 5-minute treatment with **3** at 4X MIC_{99} (8 µg/mL), colony-forming unit (CFU) titers of *S. aureus* and *P. aeruginosa* decreased more than 2 log_{10} units, an effect that was equal to the positive control compound dinitrophenol (DNP). Colistin, a cationic antimicrobial peptide that targets the membrane of Gram-negative bacteria, produced a similar effect in *P. aeruginosa*. The time-kill kinetics produced by **3** paralleled the observed membrane depolarization, suggesting a causal relationship between the rapid bactericidal effect of **3** and membrane depolarization. Bacterial outer membrane permeabilization of *P. aeruginosa* was evaluated using another fluorescent probe, 1-N-phenylnaphthylamine (NPN). Intact outer membranes exclude the hydrophobic NPN while membranes with increased fluidity and permeability allow entry of the probe, resulting in a higher fluorescent signal.[34] The aminoglycoside antibiotic gentamicin increases the outer membrane permeability of Gram-negative bacteria,[35] and was thus included

as a positive control. As shown in Figure 4C, the NPN fluorescence signal was increased rapidly in *P. aeruginosa* treated with **3** at 4X MIC$_{99}$ (32 µg/mL) and 1x MIC$_{99}$ (8 µg/mL) in a manner similar to that of gentamicin. These results suggest that **3** increases the permeability of the outer bacterial membrane. Transmission electron microscopy (TEM) was subsequently used to visualize bacterial cell morphology at high resolution and provide direct evidence of the membrane effects caused by **3**. Untreated *S. aureus* (Figure 6A) and *P. aeruginosa* (Figure 6D) possessed an intact cell envelope with both a well-defined peptidoglycan cell wall and a lipid bilayer membrane. Following treatment with **3** at 2x MIC$_{99}$ for 30-60 minutes, *S. aureus* formed numerous spherical, double layered mesosome-like structures (Figure 6B). Cellular debris of completely lysed cells was also observed after the treatment with **3** (Figure 6C). Similarly, *P. aeruginosa* treated with **3** at 2x MIC$_{99}$ (15 minutes) exhibited extensive membrane damage, as illustrated by the appearance of lysed cells and the release of cell contents (Figure 6E and 6F).

Interaction between compound 3 and antibiotics. The interactions between the membrane-targeting diamines and antibiotics with different cellular targets were investigated using checkerboard assays. The checkerboard fractional inhibitory concentration (FIC) for *S. aureus* and *P. aeruginosa* treated with **3** was determined in combination with AMC, NOR, or KAN. Based on the resulting FIC values and MIC$_{99}$ isobolograms (Figure 7), compound **3** at 0.5X MIC$_{99}$ was synergistic with KAN, causing an 8-fold reduction in the MIC$_{99}$ for KAN against *S. aureus* (from 16 to 2 µg/ml) and *P. aeruginosa* (from 64 to 8 µg/ml). By contrast, **3** was not synergistic with AMC or NOR. The synergistic interaction between **3** and KAN is consistent with previous reports that aminoglycosides interact with the bacterial cell envelope and cause membrane damage.[36, 37] Because the checkerboard method has been reported to be error prone, the synergy between **3** and KAN was verified using a time-kill kinetic assay. Eliopoulos and

Eliopoulos have suggested that an interpretation of "synergistic" requires a ≥ 2 $\log_{10}$ decrease in CFU/mL by the combination compared with the most active constituent after 24 hr, and a ≥ 2 $\log_{10}$ decrease in CFU/mL below the starting inoculum.[38] Our results show that the interaction between **3** and KAN meet these criteria (Figure 8) and confirm the synergistic relationship. *S. aureus* treated with 0.5X the MIC_{99} of compound 3 combined with 0.5X the MIC_{99} of KAN reached more than 2 $\log_{10}$ decrease in viable cell count compared with treatment with 1X the MIC_{99} of KAN alone, and to treatment at 1X the MIC_{99} of **3** alone (Figure 8A). Similarly, treatment of *P. aeruginosa* with 0.5X the MIC_{99} of **3** plus 0.5X the MIC_{99} of KAN produced the same effect (Figure 8B).

Discussion

In order to discover antibacterial agents with novel mechanisms of action, many recent studies have focused on identifying new bacterial targets or metabolic pathways. A relatively smaller number of studies have described compounds targeted to the bacterial membrane and the formation and disruption of biofilm. The cationic antimicrobial AMPs have shown promise as antibiotics that are potentially able to overcome bacterial resistance.[2] The antimicrobial action of AMPs is mediated through interaction of their positively charged structures and hydrophobic moieties with the bacterial cell membrane. In this study, we investigated the antibacterial properties of functionalized diamines containing positive moieties that can mimic the AMPs and confirmed that these compounds have potent bactericidal activity by depolarizing membrane potential and disrupting bacterial membrane integrity. The importance of the positive charges in mediating this activity is underscored by the fact that compounds **13-16**, which lack the central nitrogen moieties and thus are uncharged at physiological pH, have no detectable activity as

compared to diamines with the same internal chain length. Both Gram-positive *S. aureus* and Gram-negative *P. aeruginosa* and *E. coli* bacteria were susceptible to the bactericidal effect of the synthetic diamine **3**, which was chosen for further investigation. Importantly, compound **3** showed impressive activity against drug-resistant MRSA, dispersed biofilm in log phase and stationary-phase bacteria, and produced a synergistic interaction with the aminoglycoside antibiotic KAN. These effects were accompanied by a desirable selectivity index between the human cells evaluated in this study and bacteria.

Although the precise mechanism of action of antibacterial diamines such as **3** has not been fully elucidated, our findings strongly suggest that the Gram positive and Gram negative bacterial membrane is the primary target for these diamines. The interaction between diamines related to **3** and the bacterial membrane is likely mediated by an electrostatic interaction between the negatively charged bacterial membrane and the positively charged compounds. Binding of **3** to the membrane dissipated cell membrane potential, increased membrane permeability, and led to cell content release and lysis. Transmission electron microcopy provided the most direct evidence of the membrane disrupting action of **3**; cells treated with **3** formed abnormal membranous structures and exhibited extensive cell membrane damage. Preliminary structure activity relationship experiments demonstrate that analogues of **3** lacking charged central nitrogens were not effective against *S. aureus* and *P. aeruginosa*. While the positive charge of the compound is required for activity, it is also likely that this charge contributes to the cytotoxicity observed with these analogues at higher concentrations.[20, 39] However, this toxicity can be effectively managed when compounds related to **3** are used at lower doses as antimicrobial agents.[40, 41] Hit-to-lead optimization of antimicrobial diamines such as **3** is an ongoing concern in our laboratories.

Classical antibiotics are effective in killing actively dividing cells, but they are ineffective against bacteria that are not dividing, a phenomenon known as non-inherited antibiotic resistance.[24] Membrane-active compounds can overcome this problem and are promising antibacterial drug candidates, since both actively dividing cells and drug-resistant dormant cells require an intact membrane to stay viable.[42] Consistent with its membrane-targeting activity, compound **3** exhibited bactericidal activity against both exponential phase and stationary phase bacteria. Interestingly, the stationary phase cells were more susceptible to **3** than the exponential phase cells (Figure 3). The increased sensitivity of stationary phase cells may be due to changes in bacterial surface charges between the two growth phases. For example, the increased negative charge in the stationary-phase *S. aureus* membrane[43] may enhance the attraction of **3**, causing increased sensitivity. However, the precise mechanism of this increased sensitivity remains to be fully elucidated. The Gram-negative outer membrane is also highly negatively charged. Stationary-phase *P. aeruginosa* secretes membrane vesicles with more negative surface charges than log phase cells,[44] suggesting increased negative charges on the outer membrane. Finally, *E. coli* MG1655 also shows increased negative charge on the outer membrane as cells enter stationary phase.[45]

As outlined above, results from checkerboard assays demonstrated a synergistic interaction between **3** and KAN (Table 4 and Figures 7 and 8). The molecular basis for synergistic interactions is complex, however, synergy often occurs between compounds targeting overlapping pathways.[46] In addition to inhibiting protein synthesis, cationic aminoglycosides interact with the outer membrane of Gram-negative bacteria by displacing essential metal cations.[37] Aminoglycosides bind to outer membrane lipopolysaccharides, form transient small holes, and increase outer membrane permeability.[35-37] The cationic diamine 3 also increased

outer membrane permeability (Figure 4), suggesting that it may have a similar effect on the outer

membrane permeability as the aminoglycosides.

Conclusion. In conclusion, this report illustrates the synthetic diamines, which contain

positively charged structures, not only hold promise as potent broad-spectrum bactericidal

antibiotics, but also can be used in combination to increase the efficacy of other antibiotics by

increasing outer membrane permeability.

Experimental

Synthesis. All reagents and dry solvents were purchased from Aldrich Chemical Co. (Milwaukee, WI), Sigma Chemical Co. (St. Louis, MO), VWR (Radnor, PA) or Fisher Scientific (Chicago, IL) and were used without further purification except as noted below. Pyridine was dried by passing it through an aluminum oxide column and then stored over KOH. Triethylamine was distilled from potassium hydroxide and stored in a nitrogen atmosphere. Dry methanol, ethyl acetate, tetrahydrofuran, dimethyl formamide and hexane were prepared using a Glass Contour Solvent Purification System (Pure Process Technology, LLC, Nashua, NH). Routine chromatographic purification on silica gel was performed on a Teledyne Isco CombiFlash Rf200. Preparative scale chromatographic procedures were carried out using a CombiFlash Rf200 chromatography system (Teledyne-Isco, Lincoln, NE)fitted with silica gel 60 cartridges (230-440 mesh). Thin layer chromatography was conducted on Merck precoated silica gel 60 F-254. Ion exchange chromatography was conducted on Dowex1X8-200 anion exchange resin.

All ^{1}H- and ^{13}C-NMR spectra were recorded on a Varian Mercury 400 mHz spectrometer, and all chemical shifts are reported as δ values referenced to TMS or DSS. Splitting patterns are indicated as follows: s, singlet; d, doublet; t, triplet; m, multiplet; br, broad peak. In all cases, ^{1}H-NMR, ^{13}C-NMR and MS spectra were consistent with assigned structures. Mass spectra were recorded by LC/MS on a Waters autopurification liquid chromatography with a model 3100 mass spectrometer detector. Prior to biological testing procedures, all compounds were determined to be >95% pure by UPLC chromatography (95% H_2O/5% acetonitrile to 20% H_2O/80% acetonitrile over 10 minutes) using a Waters Acquity H-series ultrahigh-performance liquid chromatography fitted with a C18 reversed-phase column (Acquity UPLC BEH C18 1.7

M, 2.1 X 50 mm). Experimental details and analytical data for compounds **1-12** appear in previous publications.[18, 40]

1,11-bis-[(3,3-diphenylpropyl)ureido]undecane (**13**). A 0.255 g (1.07 mmol) portion of 3,3-diphenylpropyl-1-isocyanate **23** in 10 mL of dichloromethane was slowly added to 0.100 g (0.537 mmol) of 1,11-diaminoundecane **22** in 10 mL of dichloromethane with stirring at 0°C. The reaction mixture was stirred for 15 min at 0°C, warmed to room temperature and allowed to stir overnight. Completion of the reaction was verified by TLC, the solvent was evaporated and the crude product was chromatographed on silica gel 5% ethyl acetate in hexane to afford 0.634 g of pure **13** (89.6%) as a white, crystalline solid. ^{1}H NMR (CDCl$_3$): δ 7.23 (m, 20H), 4.71 (s, 4H), 3.92 (t, 2H, J=19 Hz), 3.12 (t, 4H, J=17 Hz), 3.04 (t, 4H, J=19 Hz), 2.24 (q, 4H, J=19 Hz), 1.40 (m, 4H, J=15 Hz), 1.24 (m, 14H, J=12 Hz); ^{13}C-NMR (CDCl$_3$): δ 162.1, 147.3, 131.7, 128.6, 125.3, 42.8, 36.6, 29.7, 28.2, 26.4; MS calculated 660.44, found 661.89 ([M+1]$^{+}$).

1,12-bis-[(1,1-diphenylmethyl)ureido]dodecane (**14**).Compound **14** was prepared exactly as described for compound **13** above from 1,12-diaminododecane **24** and 1,1-(diphenyl)methyl-1-isocyanate **25** (0.978 g, 73.2%) as a white amorphous solid. ^{1}H NMR (CDCl$_3$): δ 7.73 (m, 20H), 6.41 (s, 2H), 5.85 (s, 2H), 5.80 (s, 2H), 3.83 (t, 4H, J=17 Hz), 1.90 (m, 4H, J=14 Hz), 1.75 (m, 16H, J=19 Hz); ^{13}C-NMR (CDCl$_3$): δ 163.4, 143.3, 130.1, 127.9, 124.3, 62.2, 39.7, 30.5, 29.6, 29.1, 26.6; MS calculated 618.39, found 619.77 ([M+1]$^{+}$)

1,12-bis-[(1,1-diphenylmethyl)thioureido]dodecane (**15**). Compound **15** was prepared exactly as described for compound **13** above from 1,12-diaminododecane **24** and 1,1-(diphenyl)methyl-1-isothiocyanate **26** (0.980 g, 68.4%) as a white amorphous solid. ^{1}H NMR (CDCl$_3$): δ 7.31 (m, 20H), 6.40 (s, 2H), 5.61 (s, 2H), 5.30 (s, 2H), 3.44 (t, 4H, J=19 Hz), 3.41 (m, 4H, J=13 Hz), 1.52 (m, 4H), 1.40 (m, 4H), 0.85 – 1.28 (m, 8H); ^{13}C-NMR (CDCl$_3$): δ 183.2, 142.6, 129.7, 128.8, 125.0, 63.7, 46.6, 31.2, 28.4, 28.5; MS calculated 650.35, found 651.78 ([M+1]$^+$)

1,12-bis-[(2,2-diphenylethyl)ureido]dodecane (**16**). Compound **16** was prepared exactly as described for compound **13** above from 1,12-diaminododecane **24** and 2,2-(diphenyl)ethyl-1-isocyanate **27** (1.1 g, 81.2%) as a white amorphous solid. ^{1}H NMR (CDCl$_3$): δ 7.28 (m, 20H), 5.36 (s, 4H), 4.21 (m, 2H, J=14 Hz), 3.75 (d, 4H, J=17 Hz), 3.59 (m, 4H, J=12 Hz), 1.41 (m, 4H, J=19 Hz), 1.24 (m, 16H); ^{13}C-NMR (CDCl$_3$): δ 159.9, 140.8, 128.7, 128.4, 125.5, 44.2, 40.7, 39.3, 28.5, 27.9, 26.4; MS calculated 646.42, found 647.85 ([M+1]$^+$)

Strains and growth medium. Staphylococcus aureus strain RN4220 (ATCC No. 35556), *Pseudomonas aeruginosa* strain PA01 (ATCC No. 47085), and *Escherichia coli* strain ANS1[47] were grown in Luria-Bertani (LB) medium. Methicillin-resistant *Staphylococcus aureus* (MRSA) clinic isolates were grown in Mueller-Hinton (MH) medium. Human kidney cells HEK293T/17 (ATCC CRL11268) and lung cells A549 (ATCC CCL-185) were grown in Dulbecco's minimum essential medium (DMEM) with 10% heat inactivated fetal bovine serum (FBS). Nematode *Caenorhabditis elegans* strain N2 were obtained from the Caenorhabditis Genetics Center at University of Minnesota and worms were grown in nematode growth medium (NGM) for toxicity experiments.

MIC$_{99}$ assay for S. aureus strain RN4220, P. aeruginosa strain PA01 and E. coli strain ANS1. The MIC$_{99}$ assay for the antibacterial activity of each test compound was performed by the micro-broth dilution method of the Clinical and Laboratory Standards Institute of America. MIC$_{99}$ was defined as the lowest compound concentration at which bacterial growth was completely inhibited after overnight incubation in a 96-well plate (Nunc) at 37°C without shaking. MIC$_{99}$s were tested three times independently and were presented as averages ± standard deviation (SD).

MIC assay for methacillin-resistant S. aureus (MRSA). Strains were isolated from Medical University of South Carolina ICU rooms with high touch objects fabricated using US-EPA registered antimicrobial copper alloys in concert with the studies described.[48, 49] Briefly, surfaces of 100 cm^2 were sampled once a week with wipes using uniform pressure and motion, 5 strokes horizontally and 5 strokes vertically, for a total of 10 strokes. MRSA isolates were recovered from MRSA-Chromagar and were then subjected to PFGE with subsequent SPA typing.[50] The isolates were stored at -80°C until they were re-animated via cultivation on Mueller-Hinton (MH) broth for antimicrobial susceptibility testing. MIC$_{99}$ values of test compounds against MRSA strains were determined using the micro-broth dilution method described above. Overnight cultures of five MRSA strains in MH broth were diluted in fresh medium. Ten microliters of diluted cell suspension were used to inoculate 96-well plates containing 100 μl of serially diluted compounds to yield ~10^5 CFU/ml. The plates were incubated at 37°C for 24 hours before cell density was measured by absorbance at 620 nm using a Thermo MultiSkan FC plate reader.

Time-kill assay against exponential phase and stationary phase cells. 10 ml overnight cultures of strain RN4220 or PA01 were harvested, then washed and re-suspended in PBS to their initial volume, and diluted 100 fold in LB or 1% PBS, to mimic the exponential phase or stationary phase cells respectively (27). Antibiotic ampicillin (AMP), kanamycin (KAN) and norfloxacin (NOR) or compound **3** was added to 4x MIC_{99}. The cultures were incubated at 37°C with shaking at 200 rpm. At 0, 2, 4, and 6 hr time points, viable bacterial densities were estimated by diluting, plating, and counting the colony-forming unit (CFU) on LB agar plates.

Biofilm inhibition assay. Biofilm inhibition experiment was performed with slight modification as mentioned before.[51] *P. aeruginosa* PA01 was grown in LB for 2 h with shaking at 37°C, and then diluted 1:1000 in 10% LB-PBS to reach a concentration of 3×10^5 CFU/ml. The first step of the assay is to fill each well of the clear-bottomed 96-well plate (Nunc) with 25 μl of medium containing the culture inoculums. 50 μl of serial diluted compounds were added into each well and 10% LB-PBS was tested as the control. Then the 96-well plate was covered with the transferable solid-phase (TSP) pegged-lids (Nunc) and the plate was incubated at 37°C for 24 h without shaking. After 24 h incubation, biofilms formed on the pegged lid were washed twice by submerging the lid into a basin of sterilized water and then stained with 0.1% crystal violet (CV) in a new 96-well plate for 15 min with gently shaking. After this, the excess CV was washed off with sterilized water. The CV maintained in the biofilms was eluted into 150 μl 95 % ethanol and the solublized CV was quantitated spectrophotometrically at 600 nm using a Biotek Synergy HT plate reader.

To investigate the dispersal effect of the diamines, we grew the biofilms on the pegged lid without test compounds for 24 h as described above. And then, biofilms formed on the pegged lid were washed twice by submerging the lid into a basin of sterilized water. 100 μl of serial diluted compounds or 10% LB-PBS were added into a new 96-well plate. Then the pegged lid with biofilms was transferred into the 96-well plate, and then incubated at 37°C for other 24 h. The biofilms maintained in the pegged lid were measured as described above. 300 μM EDTA was used as the positive control for biofilm dispersal.

Cytoplasmic membrane depolarization assay. The membrane depolarization activity of compound **3** was determined using a fluorescent probe 3, 3'-dipropylthiacarbocyanine $DiSC_3$-5 (Sigma), which is sensitive to membrane potential.[52] Briefly, cells from log phase culture (OD_{600}~ 0.7) were harvested, washed, and re-suspended in 5mM HEPES buffer pH=7.2 containing 5 mM glucose and 100 mM KCl to an OD_{600} of 0.05. The cell suspension was incubated with 0.4 μM $DiSC_3$-5 in darkness, until a stable and approximately 90% reduction in fluorescence signal was reached. The reduction of the probe signal was due to the quenching in the cell with an intact membrane potential. After compound **3** was added (at 4x or 1x MIC_{99} concentration), changes in fluorescence due to the collapse of the cytoplasmic membrane potential were continuously recorded at 25°C using a fluorophotometer (Photon Technology), with an excitation wavelength of 622 nm and an emission wavelength of 670 nm. Compound **3** exhibited no fluorescence disturbance of the probe signal. 2,4-dinitrophenol (DNP) (Acros), which was a known inhibitor that depolarized the membrane potential and was used as a positive control.[53]

Outer membrane permeabilization assay. The effect of compound 3 on the permeability of the outer membrane of *P. aeruginosa* strain PA01 was determined by the 1-N-phenylnapthyl-amine (NPN) (Acros) assay described previously.[35, 54] Cells from strain PA01 culture at OD_{600}~0.5 were harvested, washed, and re-suspended in 5 mM HEPES buffer pH=7.2, NPN was added to the cell suspension to reach 10 μM final concentration, and sodium azide was added to reach 10 mM final concentration. After gently mixing, the cell suspension was incubated at room temperature for 30 minutes in darkness. Compound **3** at 4x or 1x MIC_{99} concentration was added, and changes of NPN fluorescence were continuously recorded at the excitation wavelength of 350 nm and emission wavelength of 420 nm. Gentamicin is a known inhibitor that increases outer membrane permeability[35] and was used as a positive control at 4x MIC_{99} concentration.

Transmission electron microscopy. Membrane damage caused by compound **3** was visualized with transmission electron microscopy. Bacteria were grown to the early exponential phase in LB (OD_{600} ~ 0.4) and treated with compound 3 at 2x MIC_{99} concentration for 15-60 min. Cells were harvested, washed twice with phosphate buffer, and then fixed with 2.5% glutaraldehyde. Samples were prepared and analyzed by transmission electron microscopy as described (24).

Drug interaction by the checkerboard MIC_{99} assay. The interactions between compound **3** and three different classes of antibiotics: ampicillin (AMP), kanamycin (KAN) and norfloxacin (NOR) were determined by the checkerboard MIC_{99} method as previously described (25, 26). Briefly, compound **3** was serially diluted along the ordinate of a 96-well plate while the antibiotic was serially diluted along the abscissa in 100 μl of LB. Each well was inoculated with

2×10^4 CFU in 100µl LB, and the plates were incubated at 37°C for 24 hours. The combined fractional inhibitory concentration (ΣFIC) was calculated as follows: ΣFIC = FIC A + FIC B, where FIC A is the MIC_{99} of drug A in the combination/MIC_{99} of drug A alone, and FIC B is the MIC_{99} of drug B in the combination/MIC_{99} of drug B alone. The drug combination is considered synergistic when the ΣFIC is less than 0.5, indifferent when the ΣFIC is between 0.5 to 4, and antagonistic when the ΣFIC is greater than 4 (26).

Drug interaction by the time-kill assay. The synergistic interaction between compound **3** and kanamycin was further confirmed by the time-kill assay. The test groups were: compound **3** or kanamycin alone at 1x and 0.5x the MIC_{99} and in combination at 0.5x the MIC_{99}. Strain RN4220 or PA01 was used to inoculate the culture at $1x10^6$ CFU/ml, and all cultures were incubated at 37°C with shaking at 300 rpm. At 0, 3, 6, and 24 hr time points, viable bacterial densities were estimated by diluting, plating, and colony counting on LB agar.

Hemolysis assay. Hemolytic experiments were performed as previously described.[55] Human red blood cells were purchased from Fisher and kept in 4 °C before use. Briefly, in a 96-well plate, 150 µl of red blood cell suspension was added into 50 µl of serially diluted compound **3**. The plate was incubated for 1h at 37°C without shaking. And then the plate was centrifuged at 3500 rpm for 5 min. 100 µl of the supernatant was transferred to a new 96-well plate, and A540 was measured. Triton X-100 was the positive control.

Cytotoxicity in human cells. Human embryonic kidney cells HEK293T/17 and human epithelial lung cells A549 were cultured in DMEM supplemented with 10% FBS at 37°C with

5% CO_2. The cells were seeded into tissue-culture treated 96-well plate and at a density of 10^4 cells per well. Twenty four hours post seeding, the cells were washed with sterile PBS once to remove the unattached cells, and then 100 µl serially-diluted compound **3** was added into the adherent cells and incubated for 48 hours. Cytotoxicity was determined using the MTS conversion assay (Promega) mentioned previously.[56] The concentration of the test compound that reduced the cell viability by 50% (CC_{50}) was determined by nonlinear regression analysis using GraphPad Prism4 software.

C. elegans toxicity assay. The nematode *C. elegans* has emerged as a good model for toxicology testing,[57] and was used to further determine the toxicity of diamines. Strain N2 worms were maintained on NGM agar at 22℃ between passages and synchronization. The effects of the test compound on adult worm survival and reproduction, juvenile worm development, and egg hatching were determined.[30] NGM agar containing compound 3 was prepared by mixing the compound with warm molten agar. Adult L4 and juvenile L2 worms were selected and transferred onto the test plate using a platinum wire. Eggs were gathered from maintenance plates using the synchronization protocol and transferred onto the test plate. Worms were incubated at 22°C and monitored every 12 hours for death, development, and egg production.

Statistical analysis. Values are presented as means ± standard deviations (SD) and analyzed by one-way ANOVA analysis of variance (Graphpad Prism). For all analyses, the criterion for significance was a *P* value of <0.05.

FIGURE LEGENDS

Figure 1. Structures of (bis)ureido or (bis)thioureido(N,N'-alkyl)diamines **1**-**12** and (bis)ureido or (bis)thioureidoalkanes **13**-**16**.
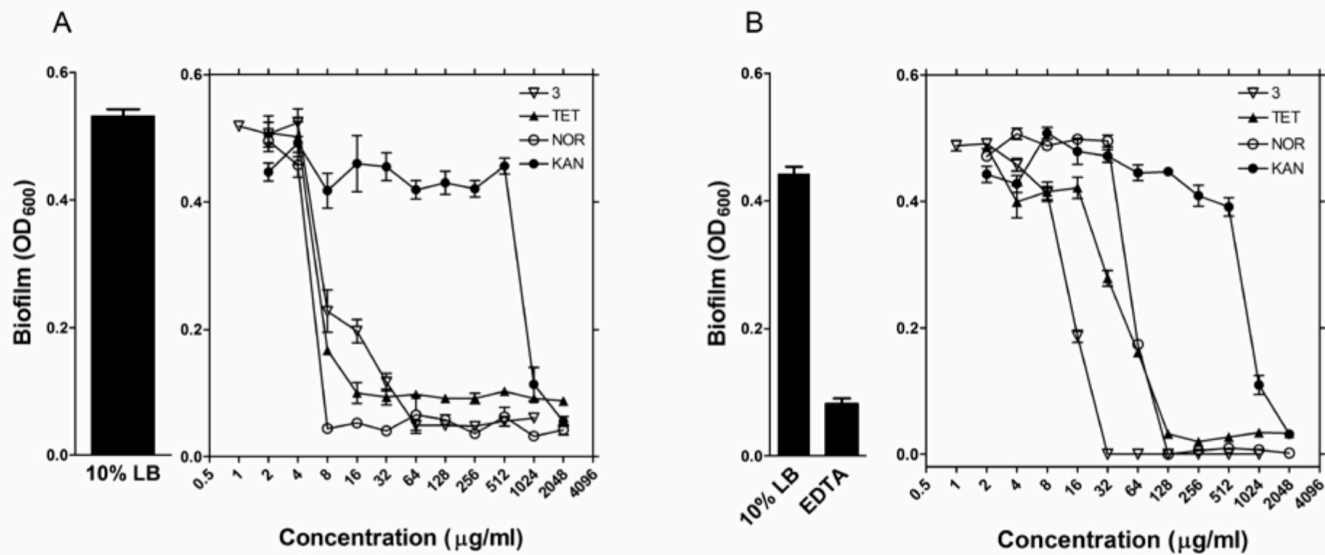
Figure 2. Effect of **3** on biofilm formation (A) and dispersal (B). The pegged-lid method was used to grow *P. aeruginosa* biofilm and the effect of **3** was compared with tetracycline (TET), norfloxacin (NOR) and kanamycin (KAN). EDTA was used as a positive control for biofilm dispersal. Each data point is the average of 3 determinations $\pm$ S.E.M.
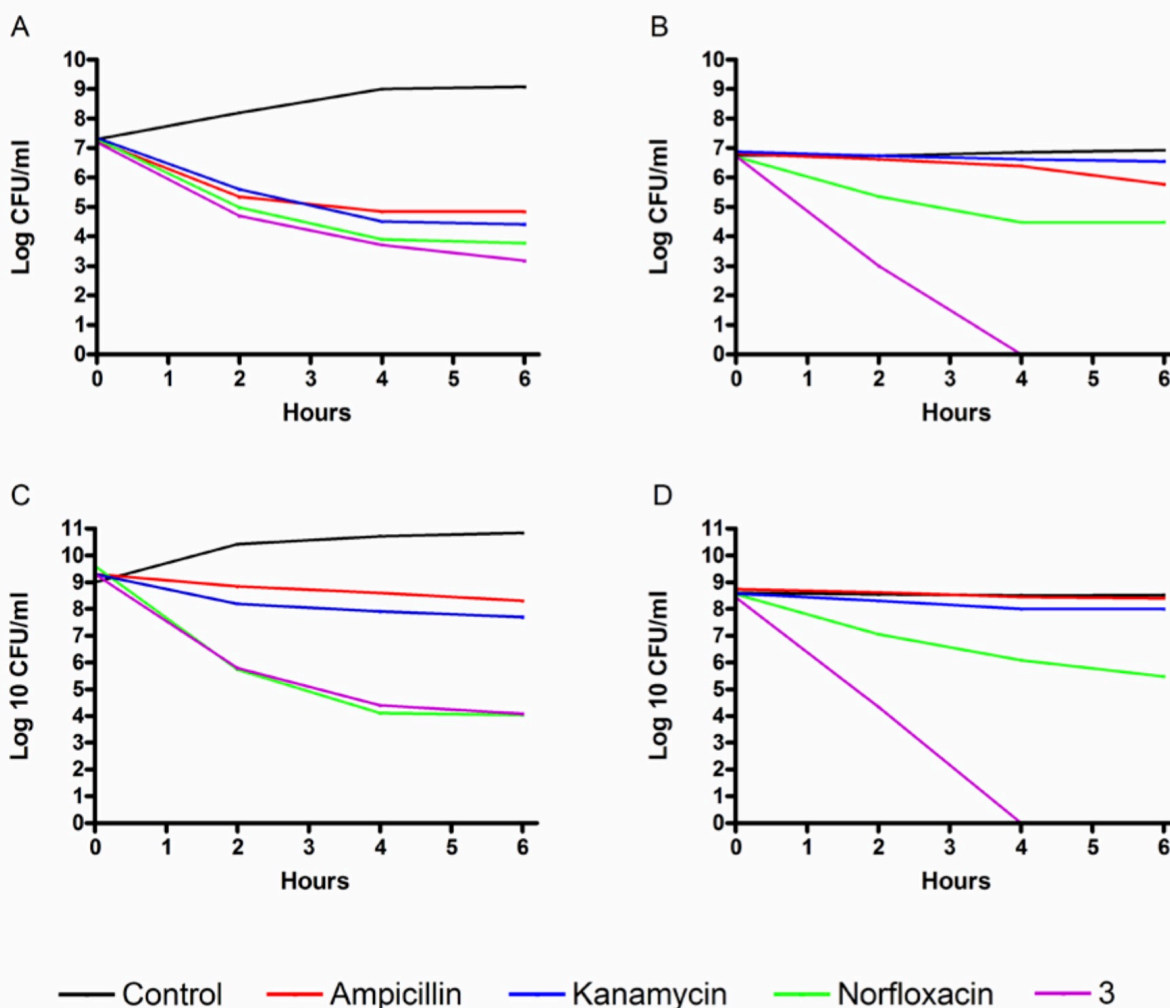
Figure 3. Viability of exponential-phase and stationary-phase bacteria in the presence of **3** or the indicated antibiotics. (**A**) exponential-phase *S. aureus* and (**B**) stationary-phase *S. aureus* were treated with antibiotics at the following concentrations: 0.5 µg/ml ampicillin sodium (1.35 µM), 32 µg/ml kanamycin sulfate (54.9 µM), 2 µg/ml norfloxacin (6.26 µM), and 8 µg/ml **3** (10.4 µM). (**C**) exponential-phase *P. aeruginosa* and (**D**) stationary-phase *P. aeruginosa* were treated with 512 µg/ml ampicillin sodium (1.38 mM), 128 µg/ml kanamycin sulfate (219.6 µM), 2 µg/ml norfloxacin (6.26 µM), and 32 µg/ml **3** (41.6 µM).

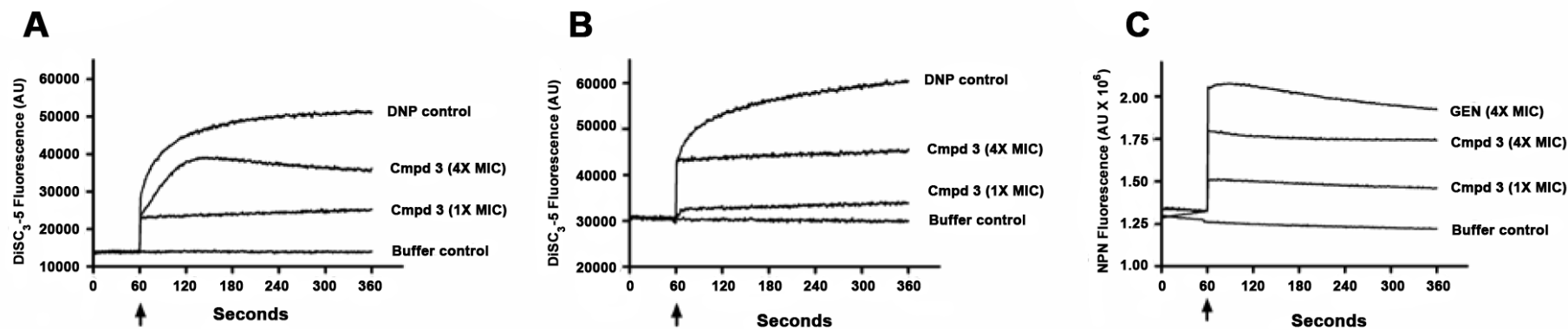**Figure 4.** (**A**) *S. aureus* cytoplasmic membrane depolarization promoted by **3**. (**B**) *P. aeruginosa c*ytoplasmic membrane depolarization promoted by **3**. (**C**) *P. aeruginosa* outer membrane permeabilization promoted by **3**. The arrow indicates the time point when the inhibitor was added. Experiments were performed three times independently and data from a representative experiment are shown.

Figure 5. (**A**) Bactericidal kinetics resulting from cytoplasmic membrane depolarization of *S. aureus* by **3**. (**B**) Bactericidal kinetics resulting from cytoplasmic membrane depolarization of *P. aeruginosa* by **3**. Each data point is the average of 3 determinations $\pm$ S.E.M.

Figure 6. Transmission electron micrographs of (**A**) untreated *S. aureus*; (**B**, **C**) *S. aureus* treated with **3** at 2x its MIC_{99}; (**D**) untreated *P. aeruginosa*; (**E**, **F**) *P. aeruginosa* treated with **3** at 2x its MIC_{99}. The arrows mark areas of membrane damage and cell content release.

Figure 7. Isobolograms showing the synergistic effects of 3 with kanamycin against *S. aureus* and *P. aeruginosa*.

Figure 8. Interaction of **3** and kanamycin (KAN) against (**A**) *S. aureus* and (**B**) *P. aeruginosa*, determined by a time-kill experiment. were performed three times independently. Each data point is the average of 3 determinations $\pm$ S.E.M.
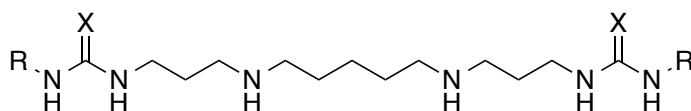
1 R = benzyl, X = S
2 R = diphenylmethyl, X = S
3 R = diphenylethyl, X = S
4 R = diphenylpropyl, X = S
5 R = phenyl, X = O
6 R = benzyl, X = O
7 R = diphenylmethyl, X = O
8 R = diphenylethyl, X = O
9 R = diphenylpropyl, X = O
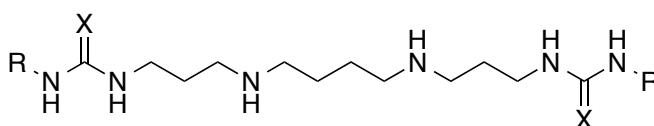
10 R = diphenylmethyl, X = S
11 R = diphenylethyl, X = O
12 R = diphenylpropyl, X = S
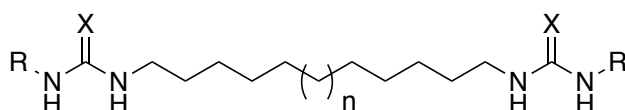
13 R = diphenylpropyl, X = O, n = 1
14 R = diphenylmethyl, X = O, n = 2
15 R = diphenylmethyl, X = S, n = 2
16 R = diphenylethyl, X = O, n = 2

Figure 1. Structures of (bis)ureido or (bis)thioureido(N,N'-alkyl)diamines **1**-**10** and (bis)ureido or (bis)thioureidoalkanes **10**-**16**.

Figure 2. Effect of **3** on biofilm formation (A) and dispersal (B). The pegged-lid method was used to grow *P. aeruginosa* biofilm and the effect of **3** was compared with tetracycline (TET), norfloxacin (NOR) and kanamycin (KAN). EDTA was used as a positive control for biofilm dispersal. Each data point is the average of 3 determinations $\pm$ S.E.M.

Figure 3. Viability of exponential-phase and stationary-phase bacteria in the presence of **3** or the indicated antibiotics. (**A**) exponential-phase *S*. *aureus* and (**B**) stationary-phase *S*. *aureus* were treated with antibiotics at the following concentrations: 0.5 μg/ml ampicillin sodium (1.35 μM), 32 μg/ml kanamycin sulfate (54.9 μM), 2 μg/ml norfloxacin (6.26 μM), and 8 μg/ml **3** (11.5 μM). (**C**) exponential-phase *P*. *aeruginosa* and (**D**) stationary-phase *P*. *aeruginosa* were treated with 512 μg/ml ampicillin sodium (1.38 mM), 128 μg/ml kanamycin sulfate (219.6 μM), 2 μg/ml norfloxacin (6.26 μM), and 32 μg/ml **3** (41.6 μM). .

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Figure 4. (**A**) *S. aureus* cytoplasmic membrane depolarization promoted by **3**. (**B**) *P. aeruginosa* cytoplasmic membrane depolarization promoted by **3**. (**C**) *P. aeruginosa* outer membrane permeabilization promoted by **3**. The arrow indicates the time point when the inhibitor was added. Experiments were performed three times independently and data from a representative experiment are shown.

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Figure 6. Transmission electron micrographs of (**A**) untreated *S. aureus*; (**B**, **C**) *S. aureus* treated with **3** at 2x its MIC_{99}; (**D**) untreated *P. aeruginosa*; (**E**, **F**) *P. aeruginosa* treated with **3** at 2x its MIC_{99}. The arrows mark areas of membrane damage and cell content release.

Figure 7. Isobolograms showing the synergistic effects of **3** with kanamycin against *S. aureus* and *P. aeruginosa*.

Figure 8. Interaction of **3** and kanamycin (KAN) against (**A**) *S. aureus* and (**B**) *P. aureginosa*, determined by time-kill experiments that were performed three times independently. Each data point is the average of 3 determinations $\pm$ S.E.M.
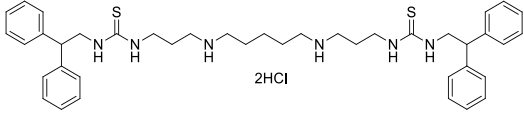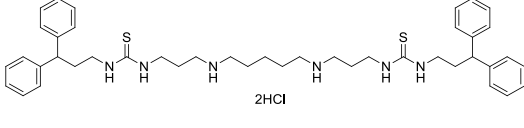
SCHEMES

Scheme 1
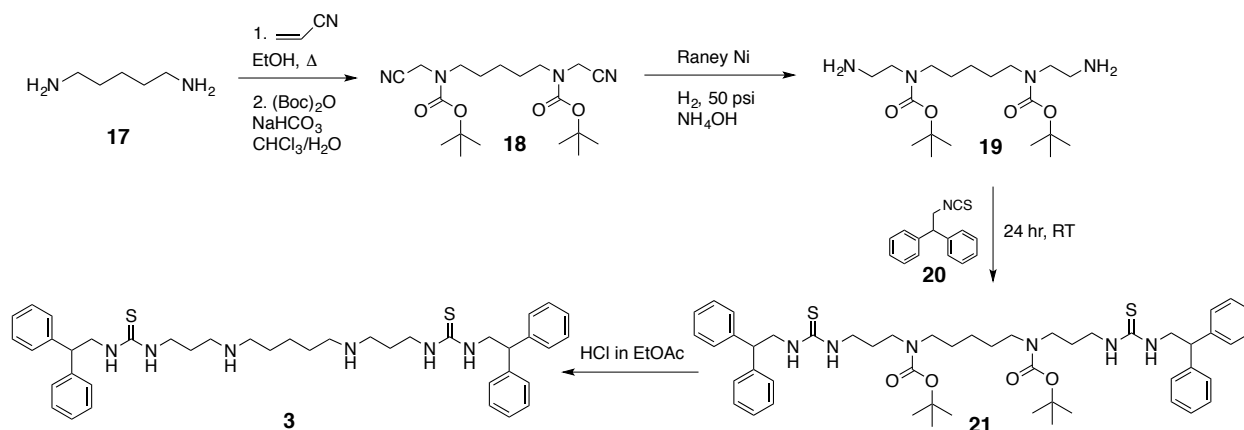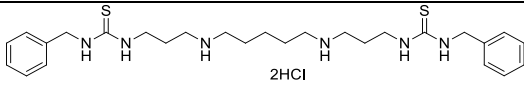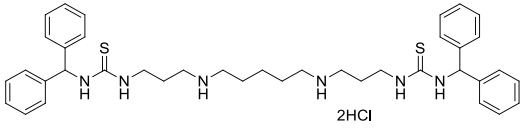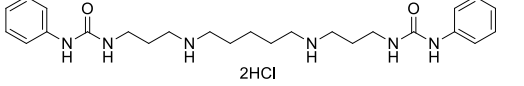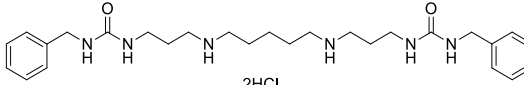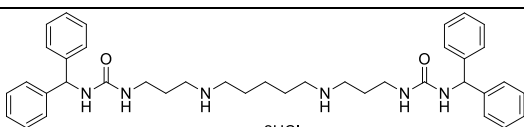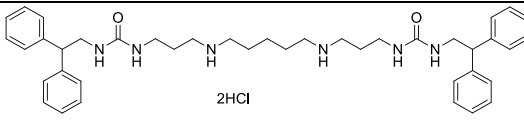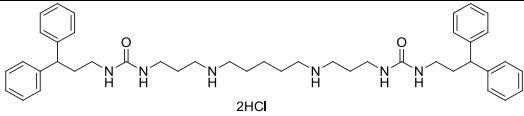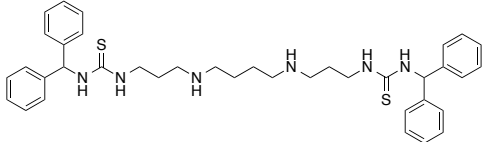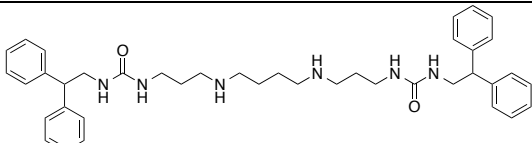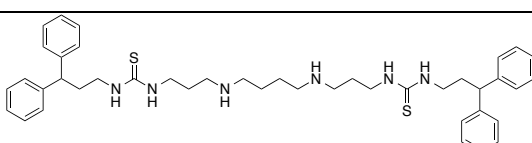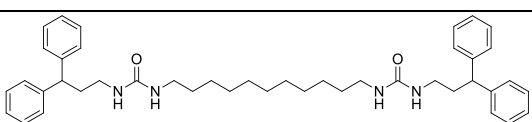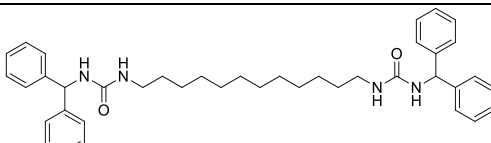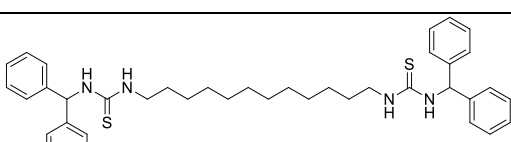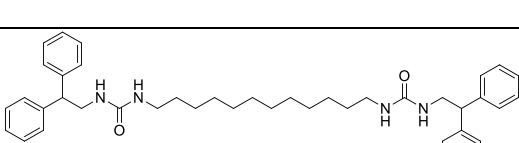
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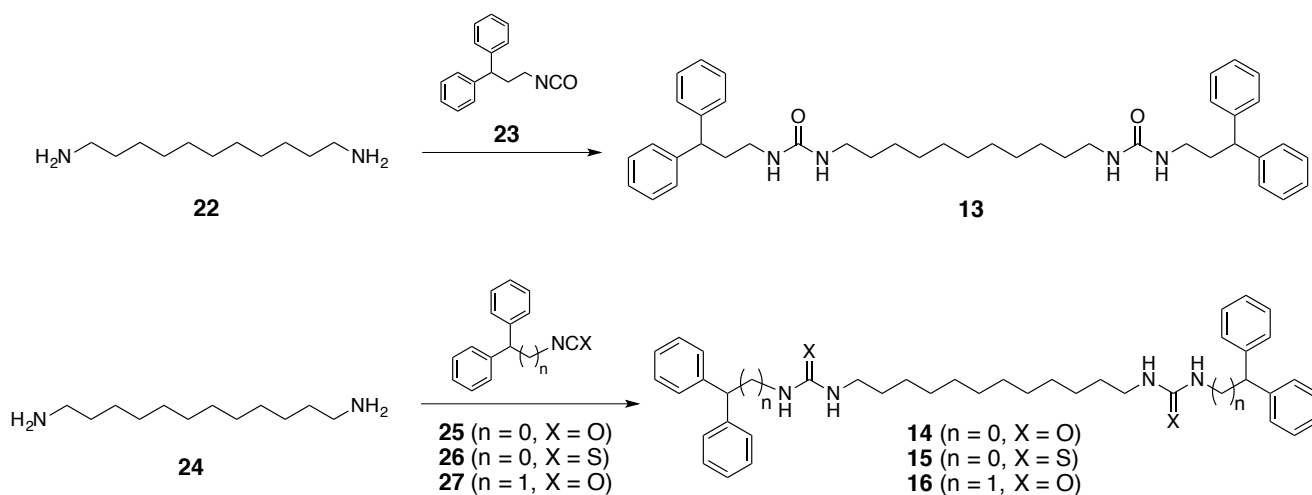
TABLES

Table 1. In vitro antibacterial activity (MIC_{99}) of diamines tested against *S. aureus* RN4220, *P. aeruginosa* PAO1 and *E. coli* ANS1. MIC_{99} values were determined three times independently and are presented as averages ± standard deviation (SD).

Compound	*Chemical Structure*	MIC_{99} (µg/ml)		
		S. aureus RN4220	*P. aeruginosa PAO1*	*E. coli ANS1*
1		32 ± 2.4	256 ± 6.3	32 ± 3.3
2		6.7 ± 2.3	13.3 ± 4.6	6 ± 2.8
3		2 ± 0.8	8 ± 1.6	2 ± 1.1
4		9.3±6.1	32 ± 5.2	10 ± 8.5
5		>256	>256	>256
6		106.7±37.3	213.3±73.9	64 ± 8.6
7		10.7±4.6	13.3±4.6	6 ± 2.8
8		18.7±12.2	26.7±9.2	4 ± 0.8

9	2HCl	13.3±4.6	26.7±9.2	6 ± 2.8
10		3.8 ± 2.9	10.4 ± 3.3	6.2 ± 1.8
11		11.3 ± 3.7	18.6 ± 5.8	4.4 ± 2.6
12		4.9 ± 1.3	29.2 ± 2.2	10.4 ± 3.7
13		>256	>256	>256
14		>256	>256	>256
15		>256	>256	>256
16		>256	>256	>256
Spermine		>512	>512	>512
Spermidine		>512	>512	>512
Ampicillin		0.125 ± 0.7	128 ± 4.3	-
Kanamycin		8 ± 1.1	32 ± 3.8	8 ± 3.3
Norfloxacin		0.5 ± 0.6	0.5 ± 02	0.25 ± 0.8

| Colistin | | >16 | < 0.125 | ND |

ND = not determined

Table 2. Effect of compound **3** on five methacillin-resistant *S. aureus* clinical isolates. MIC_{99}

values are the average of 3 determinations that in each case differed by 5% or less. MIC_{99}

average values were then rounded to the nearest whole number μg value.

Isolate ID	SPA Type	MIC_{99} (μg/mL)
10082B	1	1
10076B	2	1
30253CA	7	1
20225B	15	1
20467BA	59	1

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Table 3. Cytotoxicity of compound **3** in HEK293T human kidney embrionic cells and A549 human alveolar basal epithelial adenocarcinoma cells.

	MIC_{99}[a]	Selectivity Index[b]	
		HEK293T cells	A549 cells
S. aureus RN4220	2	16	32
P. aeruginosa PA01	8	4	8
E. coli ANS1	2	16	32
MRSA	1	32	64

[a]MIC_{99} = the concentration that inhibited bacterial growth by 99%. [b]Selectivity index = CC_{50}/MIC_{99}. The CC_{50} value of compound **3** against HEK293T cells was 32 µg/mL. The CC_{50} value of compound **3** against A549 cells was 64 µg/mL. CC_{50} = the concentration that reduced HEK293T or A549 cell viability by 50%.

Table 4. FIC values of **3** and antibiotics AMC, NOR and KAN as determined by the checkerboard method.

	FIC[a]	
	S. aureus RN4220	*P. aeruginosa* PA01
Ampicillin	16	32
Norfloxacin	4	8
Kanamycin	16	32

[a]FIC, fractional inhibitory concentration. Formula for calculating the FIC was described in the

experimental section.

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Author Contributions

‡These authors contributed equally to the work described in this manuscript.

ABBREVIATIONS

AMP, antimicrobial peptides; MRSA, methacillin-resistant Staphylococcus aureus; MIC, minimum inhibitory concentration; AMC, ampicillin; KAN, kanamycin; NOR, norfloxacin; TET, tetracycline; SI, selectivity index; CC_{50}, concentration that reduced cell viability by 50%; 5-FU, 5-fluorodideoxyuridine; NPN, 1-N-phenylnaphthylamine; TEM, transmission electron microscopy; FIC, fractional inhibitory concentration; CFU, colony-forming unit; DNP, dinitrophenol; TMS, tetramethylsilane; DSS, dioctyl sodium sulfosuccinate; LC/MS, liquid chromatography/mass spectroscopy; UPLC, ultra high performance liquid chromatography.

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REFERENCES

1.   Boucher, H. W.; Talbot, G. H.; Bradley, J. S.; Edwards, J. E.; Gilbert, D.; Rice, L. B.; Scheld, M.; Spellberg, B.; Bartlett, J.: Bad bugs, no drugs: no ESKAPE! An update from the Infectious Diseases Society of America. *Clin. Infect. Dis.* **2009,** *48* (1), 1-12.

2.   Brogden, K. A.: Antimicrobial peptides: pore formers or metabolic inhibitors in bacteria? *Nature Rev. Microbiol.* **2005,** *3* (3), 238-250.

3.   Choi, S.; Isaacs, A.; Clements, D.; Liu, D.; Kim, H.; Scott, R. W.; Winkler, J. D.; DeGrado, W. F.: De novo design and in vivo activity of conformationally restrained antimicrobial arylamide foldamers. *Proc. Natl. Acad. Sci. U. S. A.* **2009,** *106* (17), 6968-6973.

4.   Liu, D.; Choi, S.; Chen, B.; Doerksen, R. J.; Clements, D. J.; Winkler, J. D.; Klein, M. L.; DeGrado, W. F.: Nontoxic membrane-active antimicrobial arylamide oligomers. *Angew. Chem. Intl. Ed. Engl.* **2004,** *43* (9), 1158-1162.

5.   Haug, B. E.; Stensen, W.; Kalaaji, M.; Rekdal, O.; Svendsen, J. S.: Synthetic antimicrobial peptidomimetics with therapeutic potential. *J. Med. Chem.* **2008,** *51* (14), 4306-4314.

6.   Svenson, J.; Stensen, W.; Brandsdal, B. O.; Haug, B. E.; Monrad, J.; Svendsen, J. S.: Antimicrobial peptides with stability toward tryptic degradation. *Biochemistry* **2008,** *47* (12), 3777-3788.

7.   Isaksson, J.; Brandsdal, B. O.; Engqvist, M.; Flaten, G. E.; Svendsen, J. S.; Stensen, W.: A synthetic antimicrobial peptidomimetic (LTX 109): stereochemical impact on membrane disruption. *J. Med. Chem.* **2011,** *54* (16), 5786-5795.

8.   Di Martino, M. L.; Campilongo, R.; Casalino, M.; Micheli, G.; Colonna, B.; Prosseda, G.: Polyamines: emerging players in bacteria-host interactions. *Intl. J. Med. Micro.* **2013,** *303* (8), 484-491.

9.   Bottcher, T.; Kolodkin-Gal, I.; Kolter, R.; Losick, R.; Clardy, J.: Synthesis and activity of biomimetic biofilm disruptors. *J. Am. Chem. Soc.* **2013,** *135* (8), 2927-2930.

10.   Goytia, M.; Dhulipala, V. L.; Shafer, W. M.: Spermine impairs biofilm formation by *Neisseria gonorrhoeae.* *FEMS Microbiol. Lett.* **2013,** *343* (1), 64-69.

11.   Balakrishna, R.; Wood, S. J.; Nguyen, T. B.; Miller, K. A.; Suresh Kumar, E. V.; Datta, A.; David, S. A.: Structural correlates of antibacterial and membrane-permeabilizing activities in acylpolyamines. *Antimicrob. Agents Chemother.* **2006,** *50* (3), 852-861.

12.   Rabin, N.; Zheng, Y.; Opoku-Temeng, C.; Du, Y.; Bonsu, E.; Sintim, H. O.: Agents that inhibit bacterial biofilm formation. *Future Med. Chem.* **2015,** *7* (5), 647-671.

13.   Kwon, D. H.; Lu, C. D.: Polyamines increase antibiotic susceptibility in *Pseudomonas aeruginosa.* *Antimicrob. Agents Chemother.* **2006,** *50* (5), 1623-1627.

14.   Kwon, D. H.; Lu, C. D.: Polyamine effects on antibiotic susceptibility in bacteria. *Antimicrob. Agents Chemother.* **2007,** *51* (6), 2070-2077.

15.   Djouhri-Bouktab, L.; Vidal, N.; Rolain, J. M.; Brunel, J. M.: Synthesis of new 3,20-bispolyaminosteroid squalamine analogues and evaluation of their antimicrobial activities. *J. Med. Chem.* **2011,** *54* (20), 7417-7421.

16.   Djouhri-Bouktab, L.; Alhanout, K.; Andrieu, V.; Raoult, D.; Rolain, J. M.; Brunel, J. M.: Squalamine ointment for *Staphylococcus aureus* skin decolonization in a mouse model. *J. Antimicrob. Chemother.* **2011,** *66* (6), 1306-1310.

17.   Salmi, C.; Loncle, C.; Vidal, N.; Letourneux, Y.; Brunel, J. M.: New stereoselective titanium reductive amination synthesis of 3-amino and polyaminosterol derivatives possessing antimicrobial activities. *Eur. J. Med. Chem.* **2008,** *43* (3), 540-547.

18.   Nowotarski, S. L.; Pachaiyappan, B.; Holshouser, S. L.; Kutz, C. J.; Li, Y.; Huang, Y.; Sharma, S. K.; Casero, J., R.A.; Woster, P. M.: Structure-activity study for (bis)ureidopropyl- and (bis)thioureidopropyldiamine LSD1 inhibitors with 3-5-3 and 3-6-3 carbon backbone architectures. *Bioorg. Med. Chem.* **2015,** *23*, 1601-1612.

19.   Bellevue III, F. H.; Boahbedason, M.; Wu, R.; Woster, P. M.; Casero, J. R. A.; Rattendi, D.; Lane, S.; Bacchi, C. J.: Structural comparison of alkylpolyamine analogues with potent in vitro antitumor or antiparasitic activity. *Bioorg. Med. Chem. Lett.* **1996,** *6* (22), 2765-2770.

20.   Sharma, S.; Wu, Y.; Steinbergs, N.; Crowley, M.; Hanson, A.; Casero, R. A. J.; Woster, P.: (Bis)urea and (bis)thiourea inhibitors of lysine-specific demethylase 1 as epigenetic modulators. *J. Med. Chem.* **2010,** *53* (14), 5197–5212.

21.   Krossa, S.; Faust, A.; Ober, D.; Scheidig, A. J.: Comprehensive Structural Characterization of the Bacterial Homospermidine Synthase-an Essential Enzyme of the Polyamine Metabolism. *Sci. Rep.* **2016,** *6*, 19501.

22.   Ozogul, F.; Tabanelli, G.; Toy, N.; Gardini, F.: Impact of Cell-free Supernatant of Lactic Acid Bacteria on Putrescine and Other Polyamine Formation by Foodborne Pathogens in Ornithine Decarboxylase Broth. *J. Ag. Food Chem.* **2015,** *63* (24), 5828-5835.

23.   Kwon, D. H.; Lu, C. D.: Polyamines induce resistance to cationic peptide, aminoglycoside, and quinolone antibiotics in *Pseudomonas aeruginosa* PAO1. *Antimicrob. Agents Chemother.* **2006,** *50* (5), 1615-1622.

24.   Levin, B. R.; Rozen, D. E.: Non-inherited antibiotic resistance. *Nature Rev. Microbiol.* **2006,** *4* (7), 556-562.

25.   Radzishevsky, I. S.; Rotem, S.; Bourdetsky, D.; Navon-Venezia, S.; Carmeli, Y.; Mor, A.: Improved antimicrobial peptides based on acyl-lysine oligomers. *Nature Biotechnol.* **2007,** *25* (6), 657-659.

26.   Kustanovich, I.; Shalev, D. E.; Mikhlin, M.; Gaidukov, L.; Mor, A.: Structural requirements for potent versus selective cytotoxicity for antimicrobial dermaseptin S4 derivatives. *J. Biol. Chem.* **2002,** *277* (19), 16941-16951.

27.   Mahajan-Miklos, S.; Tan, M. W.; Rahme, L. G.; Ausubel, F. M.: Molecular mechanisms of bacterial virulence elucidated using a *Pseudomonas aeruginosa-Caenorhabditis elegans* pathogenesis model. *Cell* **1999,** *96* (1), 47-56.

28.   Tan, M. W.; Mahajan-Miklos, S.; Ausubel, F. M.: Killing of *Caenorhabditis elegans* by Pseudomonas aeruginosa used to model mammalian bacterial pathogenesis. *Proc. Natl. Acad. Sci. U. S. A.* **1999,** *96* (2), 715-720.

29.   Neu, A. K.; Mansson, M.; Gram, L.; Prol-Garcia, M. J.: Toxicity of bioactive and probiotic marine bacteria and their secondary metabolites in *Artemia* sp. and *Caenorhabditis elegans* as eukaryotic model organisms. *App. Environ. Micro.* **2014,** *80* (1), 146-153.

30.   Hunt, P. R.; Olejnik, N.; Sprando, R. L.: Toxicity ranking of heavy metals with screening method using adult *Caenorhabditis elegans* and propidium iodide replicates toxicity ranking in rat. *Food Chem. Toxicol.* **2012,** *50* (9), 3280-3290.

31.   Smith, C. R.; Lipsky, J. J.; Laskin, O. L.; Hellmann, D. B.; Mellits, E. D.; Longstreth, J.; Lietman, P. S.: Double-blind comparison of the nephrotoxicity and auditory toxicity of gentamicin and tobramycin. *N. Engl. J. Med.* **1980,** *302* (20), 1106-1109.

32.   Mojardin, L.; Botet, J.; Quintales, L.; Moreno, S.; Salas, M.: New insights into the RNA-based mechanism of action of the anticancer drug 5'-fluorouracil in eukaryotic cells. *PLoS ONE* **2013,** *8* (11), e78172.

33.   Lunde, C. S.; Hartouni, S. R.; Janc, J. W.; Mammen, M.; Humphrey, P. P.; Benton, B. M.: Telavancin disrupts the functional integrity of the bacterial membrane through targeted interaction with the cell wall precursor lipid II. *Antimicrob. Agents Chemother*. **2009,** *53* (8), 3375-3383.

34.   Helander, I. M.; Mattila-Sandholm, T.: Fluorometric assessment of gram-negative bacterial permeabilization. *J. Appl. Microbiol*. **2000,** *88* (2), 213-219.

35.   Hancock, R. E.; Farmer, S. W.; Li, Z. S.; Poole, K.: Interaction of aminoglycosides with the outer membranes and purified lipopolysaccharide and OmpF porin of *Escherichia coli*. *Antimicrob. Agents Chemother*. **1991,** *35* (7), 1309-1314.

36.   Peterson, A. A.; Hancock, R. E.; McGroarty, E. J.: Binding of polycationic antibiotics and polyamines to lipopolysaccharides of *Pseudomonas aeruginosa*. *J. Bacteriol*. **1985,** *164* (3), 1256-1261.

37.   Martin, N. L.; Beveridge, T. J.: Gentamicin interaction with *Pseudomonas aeruginosa* cell envelope. *Antimicrob. Agents Chemother*. **1986,** *29* (6), 1079-1087.

38.   Eliopoulos, G. M.; Eliopoulos, C. T.: Antibiotic combinations: should they be tested? *Clin. Microbiol. Rev*. **1988,** *1* (2), 139-156.

39.   Nowotarski, S. L.; Woster, P. M.; Casero, R. A., Jr.: Polyamines and cancer: implications for chemotherapy and chemoprevention. *Exp. Rev. Mol. Med*. **2013,** *15*, e3.

40.   Verlinden, B. K.; de Beer, M.; Pachaiyappan, B.; Besaans, E.; Andayi, W. A.; Reader, J.; Niemand, J.; van Biljon, R.; Guy, K.; Egan, T.; Woster, P. M.; Birkholtz, L. M.: Interrogating

alkyl and arylalkylpolyamino (bis)urea and (bis)thiourea isosteres as potent antimalarial chemotypes against multiple lifecycle forms of *Plasmodium falciparum* parasites. *Bioorg. Med. Chem.* **2015,** *23* (16), 5131-5143.

41.   Verlinden, B. K.; Niemand, J.; Snyman, J.; Sharma, S. K.; Beattie, R. J.; Woster, P. M.; Birkholtz, L. M.: Discovery of novel alkylated (bis)urea and (bis)thiourea polyamine analogues with potent antimalarial activities. *J. Med. Chem.* **2011,** *54* (19), 6624-6633.

42.   Hurdle, J. G.; O'Neill, A. J.; Chopra, I.; Lee, R. E.: Targeting bacterial membrane function: an underexploited mechanism for treating persistent infections. *Nature Rev. Microbiol.* **2011,** *9* (1), 62-75.

43.   Matsuo, M.; Oogai, Y.; Kato, F.; Sugai, M.; Komatsuzawa, H.: Growth-phase dependence of susceptibility to antimicrobial peptides in *Staphylococcus aureus*. *Microbiology* **2011,** *157* (Pt 6), 1786-1797.

44.   Tashiro, Y.; Ichikawa, S.; Shimizu, M.; Toyofuku, M.; Takaya, N.; Nakajima-Kambe, T.; Uchiyama, H.; Nomura, N.: Variation of physiochemical properties and cell association activity of membrane vesicles with growth phase in *Pseudomonas aeruginosa*. *Appl. Environ. Micro.* **2010,** *76* (11), 3732-3739.

45.   Eboigbodin, K. E.; Newton, J. R.; Routh, A. F.; Biggs, C. A.: Bacterial quorum sensing and cell surface electrokinetic properties. *Appl. Microbiol. Biotechnol.* **2006,** *73* (3), 669-675.

46.   Wolska, K. I.; Grzes, K.; Kurek, A.: Synergy between novel antimicrobials and conventional antibiotics or bacteriocins. *Polish J. Microbiol.* **2012,** *61* (2), 95-104.

47.   Jackowski, S.; Zhang, Y. M.; Price, A. C.; White, S. W.; Rock, C. O.: A missense mutation in the fabB (beta-ketoacyl-acyl carrier protein synthase I) gene confers tiolactomycin resistance to *Escherichia coli*. *Antimicrob. Agents Chemother.* **2002,** *46* (5), 1246-1252.

48.   Salgado, C. D.; Sepkowitz, K. A.; John, J. F.; Cantey, J. R.; Attaway, H. H.; Freeman, K. D.; Sharpe, P. A.; Michels, H. T.; Schmidt, M. G.: Copper surfaces reduce the rate of healthcare-acquired infections in the intensive care unit. *Infect. Control Hosp. Epidemiol.* **2013,** *34* (5), 479-486.

49.   Schmidt, M. G.; Attaway, H. H.; Sharpe, P. A.; John, J., Jr.; Sepkowitz, K. A.; Morgan, A.; Fairey, S. E.; Singh, S.; Steed, L. L.; Cantey, J. R.; Freeman, K. D.; Michels, H. T.; Salgado, C. D.: Sustained reduction of microbial burden on common hospital surfaces through introduction of copper. *J. Clin. Microbiol.* **2012,** *50* (7), 2217-2223.

50.   Shopsin, B.; Gomez, M.; Montgomery, S. O.; Smith, D. H.; Waddington, M.; Dodge, D. E.; Bost, D. A.; Riehman, M.; Naidich, S.; Kreiswirth, B. N.: Evaluation of protein A gene polymorphic region DNA sequencing for typing of *Staphylococcus aureus* strains. *J. Clin. Microbiol.* **1999,** *37* (11), 3556-3563.

51.   Junker, L. M.; Clardy, J.: High-throughput screens for small-molecule inhibitors of *Pseudomonas aeruginosa* biofilm development. *Antimicrob. Agents Chemother.* **2007,** *51* (10), 3582-3590.

52.   Wu, M.; Maier, E.; Benz, R.; Hancock, R. E.: Mechanism of interaction of different classes of cationic antimicrobial peptides with planar bilayers and with the cytoplasmic membrane of *Escherichia coli*. *Biochemistry* **1999,** *38* (22), 7235-7242.

53.   Shchepina, L. A.; Pletjushkina, O. Y.; Avetisyan, A. V.; Bakeeva, L. E.; Fetisova, E. K.; Izyumov, D. S.; Saprunova, V. B.; Vyssokikh, M. Y.; Chernyak, B. V.; Skulachev, V. P.: Oligomycin, inhibitor of the F0 part of H+-ATP-synthase, suppresses the TNF-induced apoptosis. *Oncogene* **2002,** *21* (53), 8149-8157.

54. Loh, B.; Grant, C.; Hancock, R. E.: Use of the fluorescent probe 1-N-phenylnaphthylamine to study the interactions of aminoglycoside antibiotics with the outer membrane of Pseudomonas aeruginosa. *Antimicrob. Agents Chemother.* **1984,** *26* (4), 546-551.

55. Ghosh, C.; Manjunath, G. B.; Akkapeddi, P.; Yarlagadda, V.; Hoque, J.; Uppu, D. S.; Konai, M. M.; Haldar, J.: Small molecular antibacterial peptoid mimics: the simpler the better! *J. Med. Chem.* **2014,** *57* (4), 1428-1436.

56. Leon, C. G.; Lee, J.; Bartlett, K.; Gershkovich, P.; Wasan, E. K.; Zhao, J.; Clement, J. G.; Wasan, K. M.: In vitro cytotoxicity of two novel oral formulations of Amphotericin B (iCo-009 and iCo-010) against *Candida albicans*, human monocytic and kidney cell lines. *Lipids Health Dis.* **2011,** *10*, 144.

57. Leung, M. C.; Williams, P. L.; Benedetto, A.; Au, C.; Helmcke, K. J.; Aschner, M.; Meyer, J. N.: *Caenorhabditis elegans*: an emerging model in biomedical and environmental toxicology. *Toxicol. Sci.* **2008,** *106* (1), 5-28.

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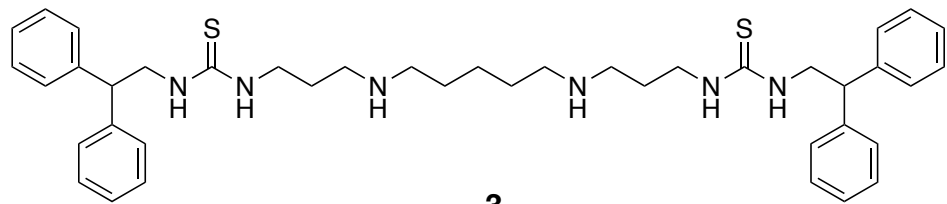
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MIC_{99} *S. aureus* RN4220 = 2 μg/mL (2.88 μM)

MIC_{99} *P. aeruginosa PA01* = 8 μg/mL (11.51 μM)

MIC_{99} *E. coli ANS1* = 2 μg/mL (2.88 μM)

MIC_{99} *MRSA* 10082B = 2 μg/mL (2.88 μM)