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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 of the cytoplasmic membrane 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.

Graphical abstract

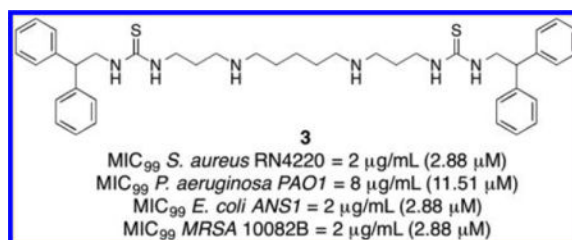
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Note

The authors declare no competing financial interest.



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 are known to act primarily by disrupting the bacterial cell membrane. Moreover, the charge–charge interaction between antibacterial peptides and bacterial cell membrane does not target intracellular bacterial proteins. Thus, bacteria find it difficult to develop resistance against antibacterial peptides.² However, antibacterial peptides 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 antibacterial peptides 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 nondividing 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 article 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)₂O, NaHCO₃, and CHCl₃/H₂O] to afford the previously described (bis)-N-Boc protected intermediate **18**.^{19,20} Compound **18** was reduced in the presence of Raney nickel (H₂, 50 psi, NH₄OH) 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 equiv 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 MIC₉₉ of compounds **1–16** was determined against a Gram-positive organism (*Staphylococcus aureus* strain RN4220) and 2 Gram-negative organisms (*Pseudomonas aeruginosa* strain PAO1 and *Escherichia coli* strain ANS1). The MIC₉₉ assay for the antibacterial activity of each test compound was performed by the microbroth dilution method of the Clinical and Laboratory Standards Institute of America. MIC₉₉ 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 (AMP), the aminoglycoside kanamycin (KAN), the fluoroquinolone 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 MIC₉₉ values between 2 and 32 µg/mL (with one exception: compound **1** had an MIC₉₉ value of 256 µg/mL against *P. aeruginosa*). The most effective of these analogues, **3**, exhibited MIC₉₉ values of 2.0, 8.1, and 2.2 µg/mL against *S. aureus* RN4220, *P. aeruginosa* PAO1, 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. On the basis of 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 is 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, amino-glycosides, quinolones, and fluorescent dyes.²³ In a subsequent study by the same group, spermine was shown to decrease the MIC values for chloramphenicol and β -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 MIC₉₉ 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 MIC₉₉ values. Also, the (bis)arylthioureido analogues were slightly more effective than the corresponding (bis)aryllureido 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 Meth-acillin-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 MIC₉₉ values of 1–2 $\mu\text{g}/\text{mL}$ against each isolate (Table 2). These MIC₉₉ values are comparable to the MIC value of **3** against *S. aureus* RN4220 (2 $\mu\text{g}/\text{mL}$, 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 $\mu\text{g/mL}$, which was comparable to the commercial antibiotics tetracycline (TET) and NOR, whereas KAN displayed no inhibitory effect below 1024 $\mu\text{g/mL}$ (Figure 2A). Interestingly, **3** had a significantly stronger effect on the dispersal of the formed biofilm (Figure 2B) when compared to that of TET, NOR, and KAN. The term drug tolerance refers to the noninherited drug resistance that develops against antibiotics in nondividing 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 nondividing 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 nondividing stationary cells (Figure 3). By contrast, the traditional antibiotics AMP, KAN, and NOR displayed strong bactericidal activity against dividing bacteria but did not kill stationary phase bacteria efficiently. When tested at bactericidal concentrations ($4\times \text{MIC}_{99}$), AMP, 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 AMP, KAN, and NOR, while remaining susceptible to **3** (Figure 3B). Following 4 h of treatment, NOR reduced the viable cell numbers only by 2 logs, while **3** eliminated all viable cells. Log phase *P. aeruginosa* responded weakly to AMP 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 AMP 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 h. These data demonstrate that the membrane-targeting diamine **3** can circumvent drug tolerance in non-dividing cells since both dividing log phase and nondividing stationary phase bacteria require intact membranes to survive.

Antibacterial Activity in Plasma

One of the major disadvantages of antibacterial peptides is their potential for proteolytic inactivation. This is reflected in the subsequent loss of antibacterial activity of antibacterial peptides 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 microbroth dilution method as described above. Following 12 h of preincubation 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 $\mu\text{g/mL}$, indicating that the compound produced selective toxicity toward

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 CC₅₀ value of cytotoxicity against a given human cell line divided by the MIC₉₉ 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. aeruginosa* 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-fluorodeoxyuridine (5FU). Gentamicin and 5FU are both toxic toward 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, worms at L1 and L4 development stages, were incubated with 64 $\mu\text{g/mL}$ 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 toward *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 antibacterial peptides 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 (DiSC₃₋₅). 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 4 \times MIC₉₉ (8 $\mu\text{g/mL}$) and 1 \times

MIC₉₉ (2 $\mu\text{g}/\text{mL}$) concentrations against *S. aureus* caused a rapid increase in the probe's fluorescence intensity (Figure 4A), indicating that the membrane potential was dissipated by the diamine. Treatment of *P. aeruginosa* by compound **3** led to a similar abrupt increase in DiSC₃₋₅ fluorescence (Figure 4B), demonstrating that 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 min treatment with **3** at 4 \times MIC₉₉ (8 $\mu\text{g}/\text{mL}$), colony-forming unit (CFU) titers of *S. aureus* and *P. aeruginosa* decreased more than 2 log₁₀ 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*-phenyl naphthylamine (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 bacteria³⁵ 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 4 \times MIC₉₉ (32 $\mu\text{g}/\text{mL}$) and 1 \times MIC₉₉ (8 $\mu\text{g}/\text{mL}$) 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 2 \times MIC₉₉ for 30–60 min, *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 2 \times MIC₉₉ (15 min) exhibited extensive membrane damage, as illustrated by the appearance of lysed cells and the release of cell contents (Figure 6E and F).

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 AMP, NOR, or KAN. On the basis of the resulting ΣFIC (Table 4) values and MIC₉₉ isobolograms (Figure 7), compound **3** at 0.5 \times MIC₉₉ was synergistic with KAN, causing an 8-fold reduction in the MIC₉₉ for KAN against *S. aureus* (from 16 to 2 $\mu\text{g}/\text{mL}$) and *P. aeruginosa* (from 64 to 8 $\mu\text{g}/\text{mL}$). By contrast, **3** was not synergistic with AMP 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 h 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 0.5× the MIC₉₉ of compound **3** combined with 0.5× the MIC₉₉ of KAN reached more than $2 \log_{10}$ decrease in viable cell count compared with the treatment with 1× the MIC₉₉ of KAN alone, and to treatment at 1× the MIC₉₉ of **3** alone (Figure 8A). Similarly, treatment of *P. aeruginosa* with 0.5× the MIC₉₉ of **3** plus 0.5× the MIC₉₉ 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 biofilms. The cationic antimicrobial antibacterial peptides have shown promise as antibiotics that are potentially able to overcome bacterial resistance.² The antimicrobial action of antibacterial peptides 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 antibacterial peptides 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 microscopy 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 noninherited 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* membrane⁴³ 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 an effect on the outer membrane permeability similar to that of the aminoglycosides.

CONCLUSIONS

In conclusion, this article illustrates that 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 SECTION

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, dimethylformamide, 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 ^1H - 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; and br, broad peak. In all cases, ^1H 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 min) using a Waters Acquity H-series ultrahigh-performance liquid chromatography fitted with a C18 reversed-phase column (Acquity UPLC BEH C18 1.7 M, 2.1×50 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. ^1H 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 ($[\text{M} + 1]^+$).

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. ^1H 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 ($[\text{M} + 1]^+$).

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. $^1\text{H NMR}$ (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). $^{13}\text{C NMR}$ (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 ($[\text{M}+1]^+$)

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. $^1\text{H NMR}$ (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). $^{13}\text{C NMR}$ (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 ($[\text{M}+1]^+$)

Strains and Growth Medium

Staphylococcus aureus strain RN4220 (ATCC No. 35556), *Pseudomonas aeruginosa* strain PAO1 (ATCC No. 47085), and *Escherichia coli* strain ANS1⁴⁷ were grown in Luria–Bertani (LB) medium. Methicillin-resistant *Staphylococcus aureus* (MRSA) clinic isolates were grown in Mueller-Hinton (MH) medium. Human kidney cells HEK293 T/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 was obtained from the Caenorhabditis Genetics Center at University of Minnesota, and worms were grown in nematode growth medium (NGM) for toxicity experiments.

MIC₉₉ Assay for *S. aureus* Strain RN4220, *P. aeruginosa* Strain PAO1, and *E. coli* Strain ANS1

The MIC₉₉ assay for the antibacterial activity of each test compound was performed by the microbroth dilution method of the Clinical and Laboratory Standards Institute of America. MIC₉₉ 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. MIC₉₉s were tested three times independently and were presented as averages \pm 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 cm² 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 reanimated via cultivation on MH broth for antimicrobial susceptibility testing.

MIC₉₉ values of test compounds against MRSA strains were determined using the microbroth dilution method described above. Overnight cultures of five MRSA strains in MH broth were diluted in fresh medium. Ten microliters of diluted cell suspension was used to inoculate 96-well plates containing 100 μL of serially diluted compounds to yield $\sim 10^5$ CFU/mL. The plates were incubated at 37 °C for 24 h 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

Ten milliliter overnight cultures of strain RN4220 or PAO1 were harvested, then washed and resuspended 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 Antibiotics AMP, KAN, and NOR or compound **3** was added to $4\times$ MIC₉₉. The cultures were incubated at 37 °C with shaking at 200 rpm. At 0, 2, 4, and 6 h time points, viable bacterial numbers were estimated by diluting, plating, and counting the colony-forming unit (CFU) on LB agar plates.

Biofilm Inhibition Assay

A biofilm inhibition experiment was performed with a slight modification as mentioned before.⁵¹ *P. aeruginosa* PAO1 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 was to fill each well of the clear-bottomed 96-well plate (Nunc) with 25 μL of medium containing the culture inoculums. Then, 50 μL of serial diluted compounds was added into each well, and 10% LB-PBS was tested as the control. The 96-well plate was covered with transferable solid-phase (TSP) pegged-lids (Nunc), and the plate was incubated at 37 °C for 24 h without shaking. After 24 h of 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 of 95% ethanol, and the solubilized 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 biofilms formed on the pegged lid were washed twice by submerging the lid into a basin of sterilized water. Then, 100 μL of serial diluted compounds or 10% LB-PBS was added into a new 96-well plate. Then, the pegged lid with biofilms was transferred into the 96-well plate and incubated at 37 °C for another 24 h. The biofilms maintained in the pegged lid were measured as described above. Three hundred micromolar 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 DiSC₃₋₅ (Sigma), which is sensitive to membrane potential.⁵² Briefly, cells from log phase culture (OD₆₀₀ \sim 0.7) were harvested, washed, and resuspended in 5 mM HEPES buffer at pH 7.2 containing 5 mM glucose and 100 mM KCl to an OD₆₀₀ of 0.05. The cell suspension was incubated with 0.4 μM DiSC₃₋₅ 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 4× or 1× MIC₉₉ 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 is a known inhibitor that depolarized the membrane potential, 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 PAO1 was determined by the 1-*N*-phenyl-naphthylamine (NPN) (Acros) assay described previously.^{35,54} Cells from strain PAO1 culture at OD₆₀₀ ~ 0.5 were harvested, washed, and resuspended in 5 mM HEPES buffer at 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 min in darkness. Compound **3** at 4× or 1× MIC₉₉ 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 permeability³⁵ and was used as a positive control at 4× MIC₉₉ 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 (OD₆₀₀ ~ 0.4) and treated with compound **3** at 2× MIC₉₉ 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 in ref 24.

Drug Interaction by the Checkerboard MIC₉₉ Assay

The interactions between compound **3** and three different classes of antibiotics, AMP, KAN, and NOR, were determined by the checker-board MIC₉₉ 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 × 10⁴ CFU in 100 μL of LB, and the plates were incubated at 37 °C for 24 h. The combined fractional inhibitory concentration (ΣFIC) was calculated as follows: ΣFIC = FIC A + FIC B, where FIC A is the MIC₉₉ of drug A in the combination/MIC₉₉ of drug A alone, and FIC B is the MIC₉₉ of drug B in the combination/MIC₉₉ 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 KAN alone at 1× and 0.5× MIC₉₉ and in combination at 0.5× MIC₉₉. Strain RN4220 or PAO1 was used to inoculate the

culture at 1×10^6 CFU/mL, and all cultures were incubated at 37 °C with shaking at 300 rpm. At 0, 3, 6, and 24 h 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 μ L of red blood cell suspension was added into 50 μ L of serially diluted compound **3**. The plate was incubated for 1 h at 37 °C without shaking, and then the plate was centrifuged at 3500 rpm for 5 min. One hundred microliters 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 HEK293 T/17 and human epithelial lung cells A549 were cultured in DMEM supplemented with 10% FBS at 37 °C with 5% CO₂. The cells were seeded into tissue-culture treated 96-well plates and at a density of 10^4 cells per well. Twenty-four hours postseeding, the wells were washed with sterile PBS once to remove the unattached cells, and then 100 μ L of serially diluted compound **3** was added into the adherent cells and incubated for 48 h. 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% (CC₅₀) 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 testing⁵⁷ and was used to further determine the toxicity of diamines. Strain N2 worms were maintained on NGM agar at 22 °C 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 L1 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 h for death, development, and egg production.

Statistical Analysis

Values are presented as the means \pm 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.

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ABBREVIATIONS

MRSA	methacillin-resistant <i>Staphylococcus aureus</i>
MIC	minimum inhibitory concentration
AMP	ampicillin
KAN	kanamycin
NOR	norfloxacin
TET	tetracycline
SI	selectivity index
CC₅₀	concentration that reduced cell viability by 50%
5-FU	5-fluorodeoxyuridine
NPN	1- <i>N</i> -phenyl-naphthylamine
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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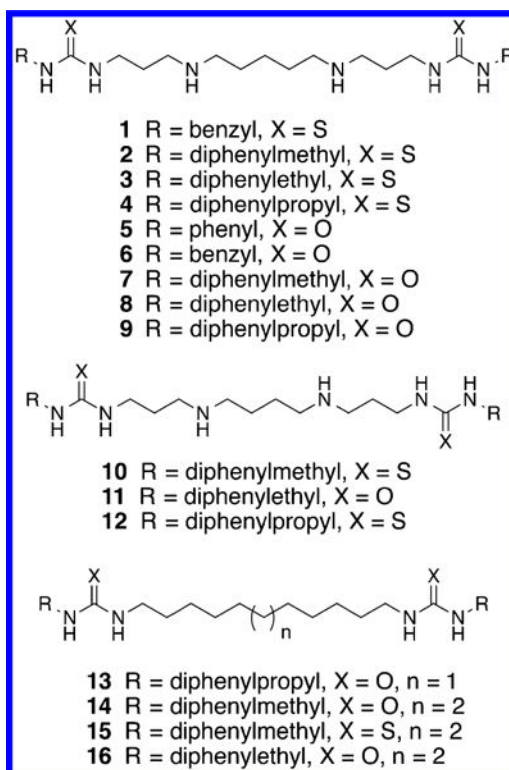


Figure 1. Structures of (bis)ureido or (bis)thioureido(*N,N'*-alkyl)-diamines **1–10** and (bis)ureido or (bis)thioureidoalkanes **10–16**.

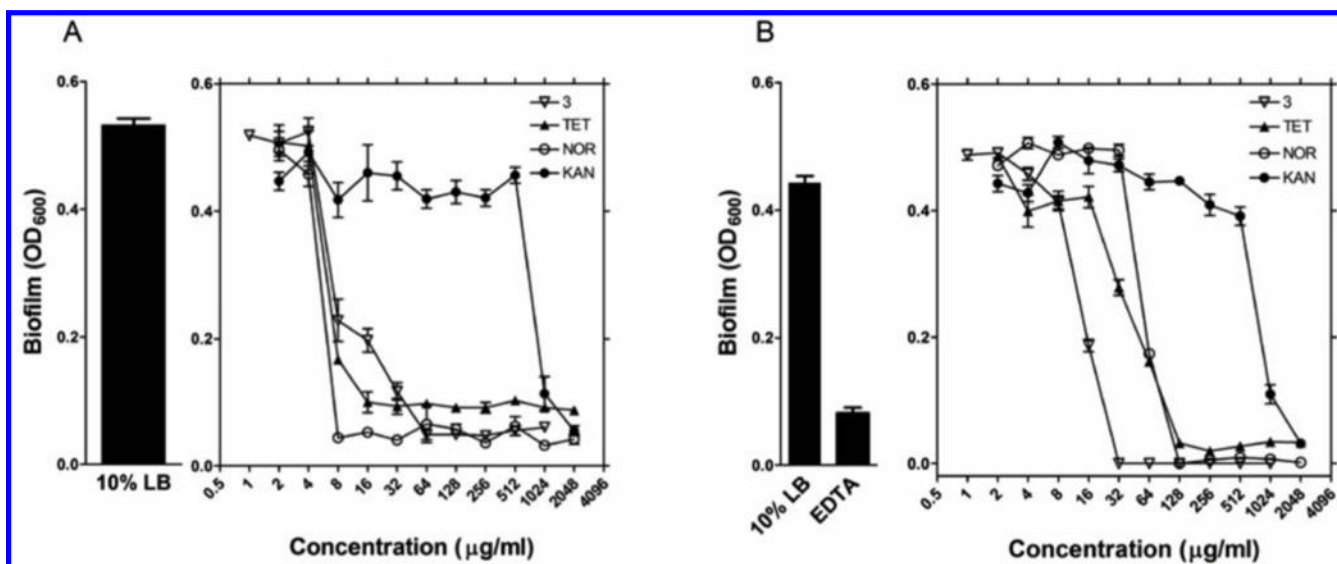


Figure 2.

Effect of **3** on biofilm formation (A) and dispersal (B). The pegged-lid method was used to grow the *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 SEM.

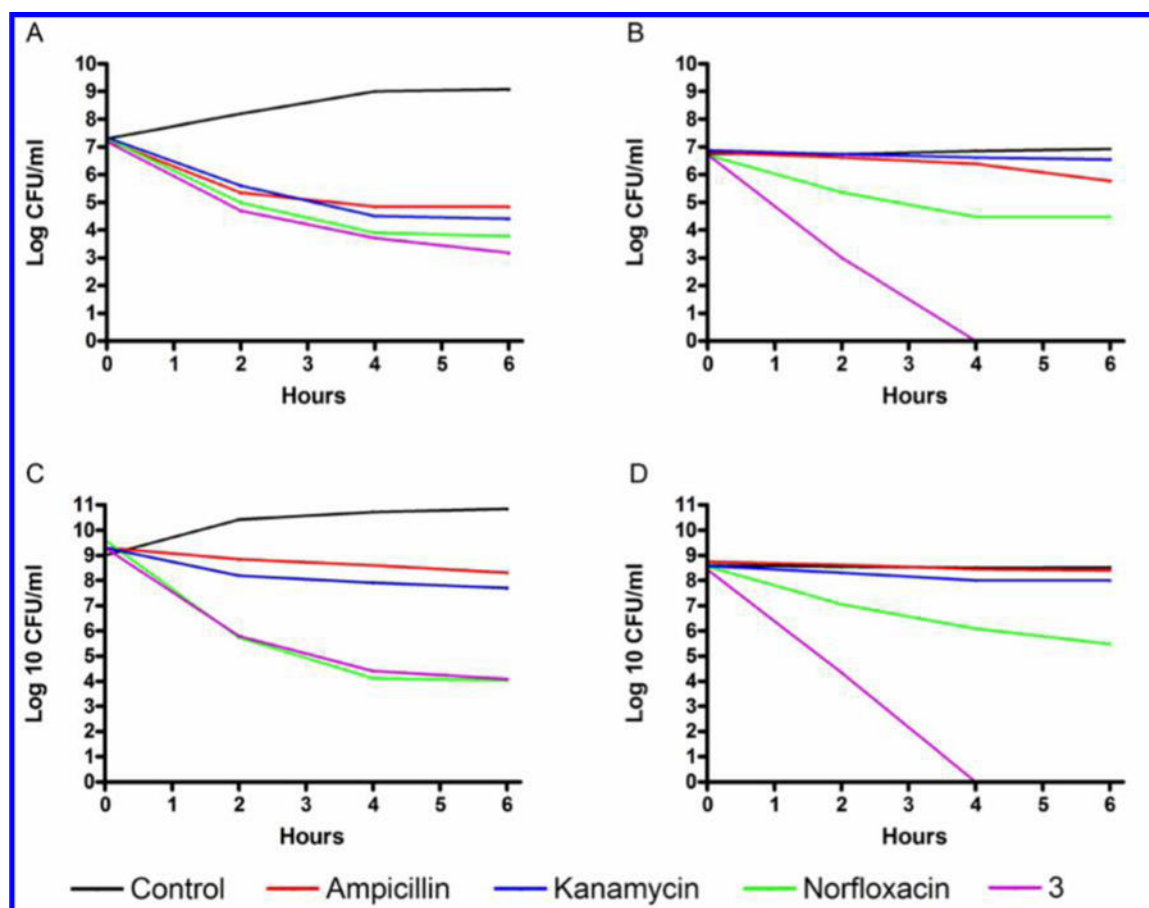


Figure 3.

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 $\mu\text{g}/\text{mL}$ ampicillin sodium (1.35 μM), 32 $\mu\text{g}/\text{mL}$ kanamycin sulfate (54.9 μM), 2 $\mu\text{g}/\text{mL}$ norfloxacin (6.26 μM), and 8 $\mu\text{g}/\text{mL}$ **3** (11.5 μM). (C) Exponential-phase *P. aeruginosa* and (D) stationaryphase *P. aeruginosa* were treated with 512 $\mu\text{g}/\text{mL}$ ampicillin sodium (1.38 mM), 128 $\mu\text{g}/\text{mL}$ kanamycin sulfate (219.6 μM), 2 $\mu\text{g}/\text{mL}$ norfloxacin (6.26 μM), and 32 $\mu\text{g}/\text{mL}$ **3** (41.6 μM).

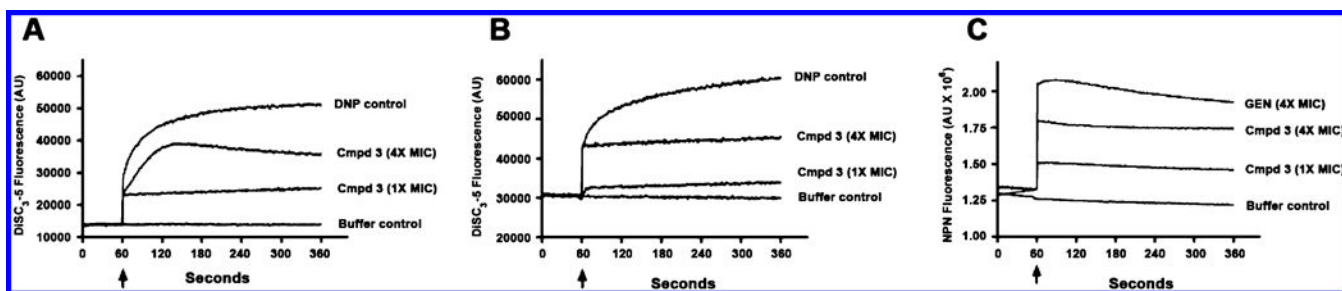


Figure 4.

(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 at which the inhibitor was added. Experiments were performed three times independently, and data from a representative experiment are shown.

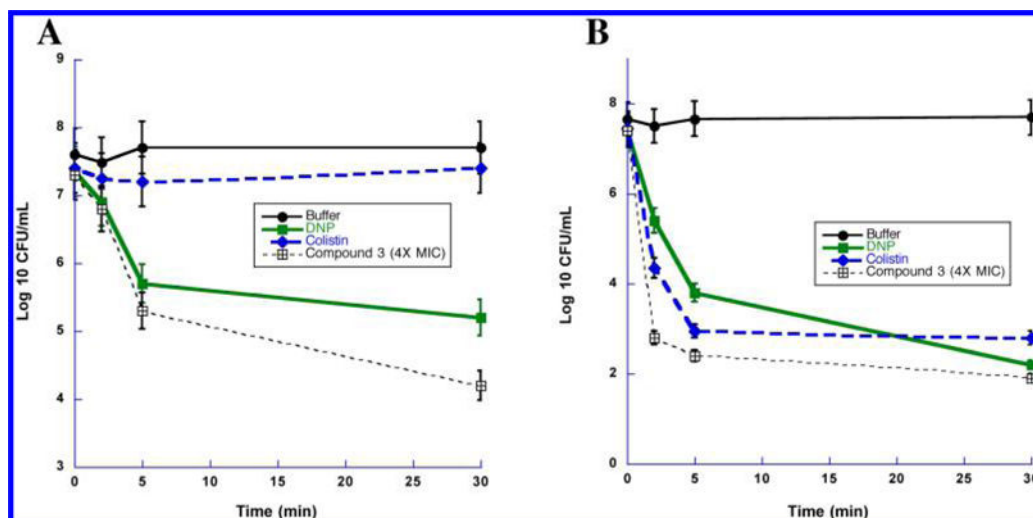


Figure 5.

(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 SEM.

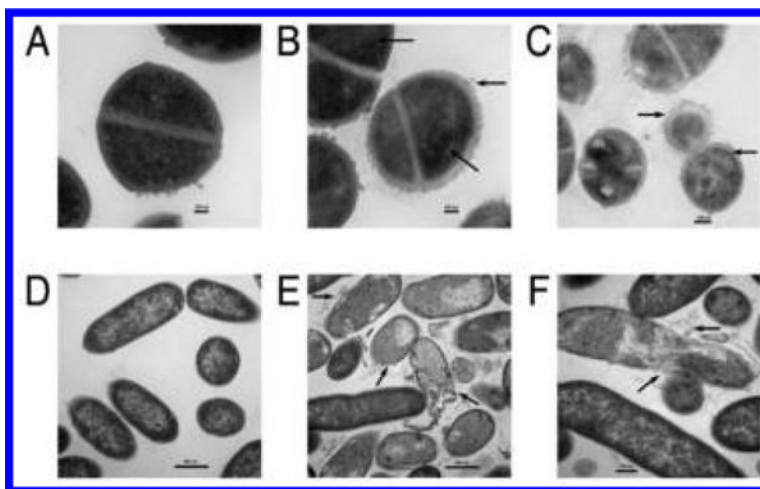


Figure 6. Transmission electron micrographs of (A) untreated *S. aureus*; (B,C) *S. aureus* treated with **3** at $2\times$ its MIC₉₉; (D) untreated *P. aeruginosa*; (E,F) *P. aeruginosa* treated with **3** at $2\times$ its MIC₉₉. The arrows mark areas of membrane damage and cell content release.

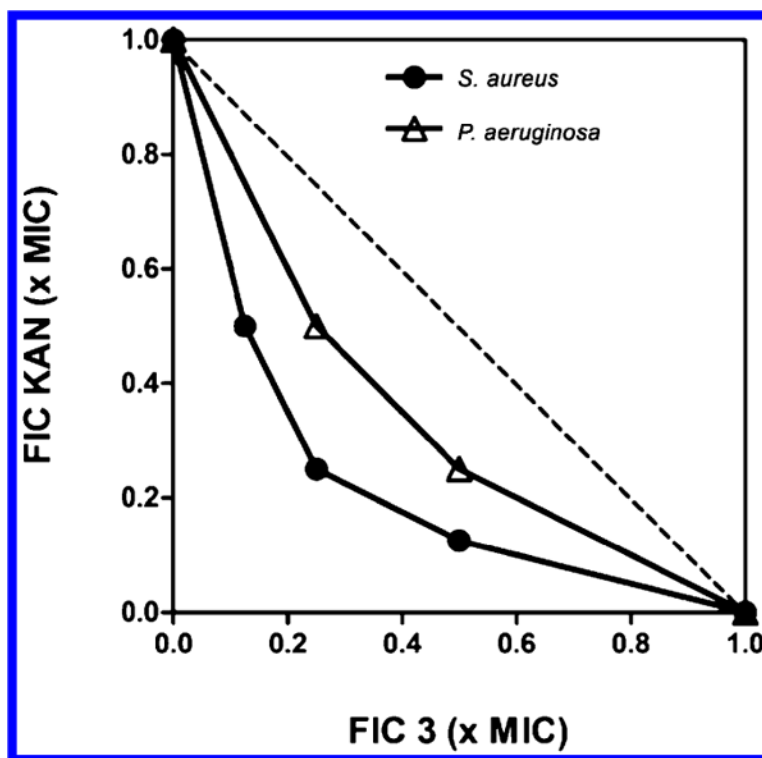


Figure 7. Isobolograms showing the synergistic effects of **3** with kanamycin against *S. aureus* and *P. aeruginosa*.

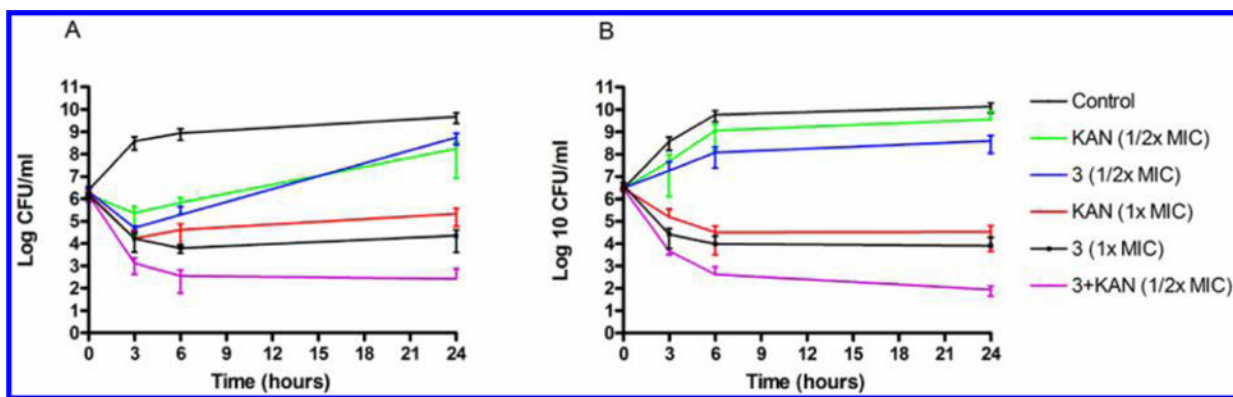
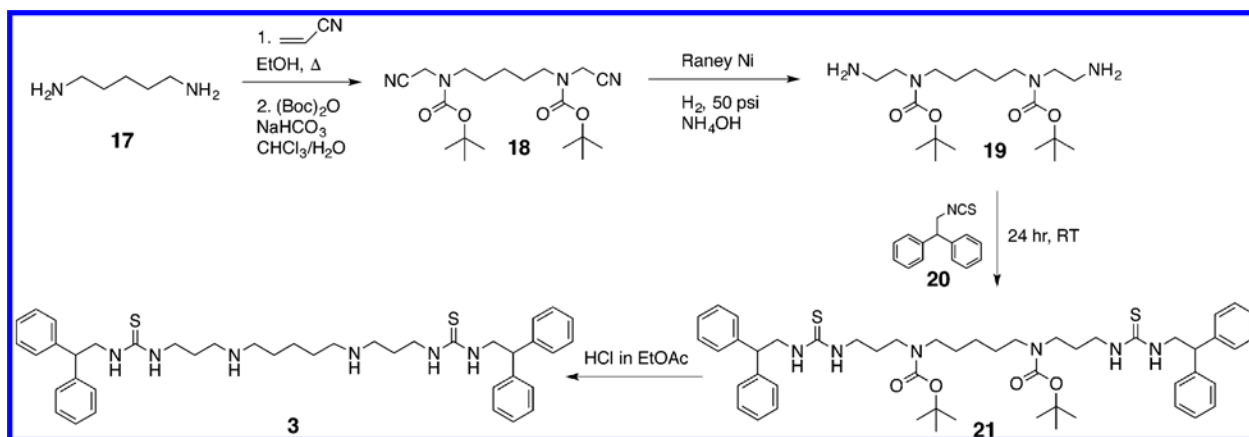
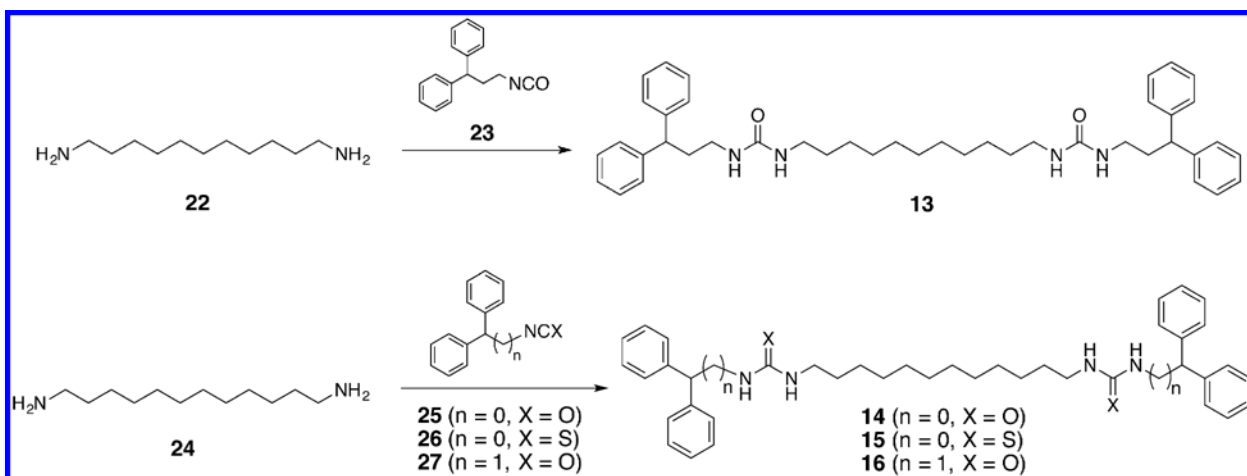


Figure 8. Interaction of **3** and kanamycin (KAN) against (A) *S. aureus* and (B) *P. aeruginosa*, determined by time-kill experiments that were performed three times independently. Each data point is the average of 3 determinations \pm SEM.



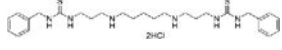
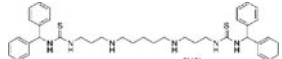
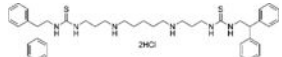
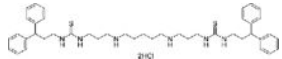
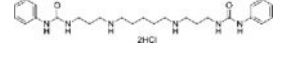
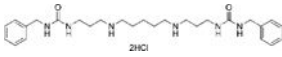
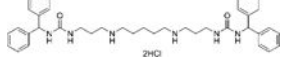
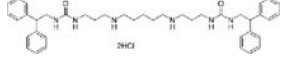
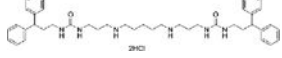
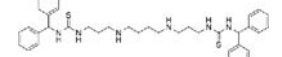
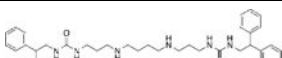
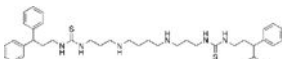

Scheme 1.

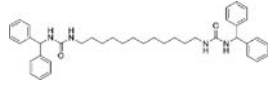

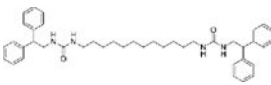


Scheme 2.

Table 1

In Vitro Antibacterial Activity (MIC₉₉) of Diamines Tested against *S. aureus* RN4220, *P. aeruginosa* PAO1, and *E. coli* ANS1^a

Compound	Chemical Structure	MIC ₉₉ (µg/ml)		
		<i>S. aureus</i> RN4220	<i>P. aeruginosa</i> PAOI	<i>E. coli</i> 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		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

Compound	Chemical Structure	MIC ₉₉ (µg/ml)		
		<i>S. aureus</i> RN4220	<i>P. aeruginosa</i> PAOI	<i>E. coli</i> ANSI
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 ± 0.2	0.25 ± 0.8
Colistin		>16	<0.125	ND

^aMIC₉₉ values were determined three times independently and are presented as averages ± standard deviation (SD). ND = not determined.

Table 2Effect of Compound 3 on Five Methacillin-Resistant *S. aureus* Clinical Isolates^a

isolate ID	SPA type	MIC ₉₉ ($\mu\text{g/mL}$)
10082B	1	1
10076B	2	1
30253CA	7	1
20225B	15	1
20467BA	59	1

^aMIC₉₉ values are the average of 3 determinations that in each case differed by 5% or less. MIC₉₉ average values were then rounded to the nearest whole number μg value.

Table 3

Cytotoxicity of Compound 3 in HEK293T Human Kidney Embryonic Cells and A549 Human Alveolar Basal Epithelial Adenocarcinoma Cells

	MIC ₉₉ ^a	selectivity index ^b	
		HEK293T cells	A549 cells
<i>S. aureus</i> RN4220	2	16	32
<i>P. aeruginosa</i> PAO1	8	4	8
<i>E. coli</i> ANSI	2	16	32
MRSA	1	32	64

^aMIC₉₉ = the concentration that inhibited bacterial growth by 99%.

^bSelectivity index = CC₅₀/MIC₉₉. The CC₅₀ value of compound **3** against HEK293T cells was 32 $\mu\text{g}/\text{mL}$. The CC₅₀ value of compound **3** against A549 cells was 64 $\mu\text{g}/\text{mL}$. CC₅₀ = the concentration that reduced HEK293T or A549 cell viability by 50%.

Table 4

FIC Values of 3 and Antibiotics AMP, NOR, and KAN As Determined by the Checkerboard Method

	Σ_{FIC}^a	
	<i>S. aureus</i> RN4220	<i>P. aeruginosa</i> PAO1
ampicillin	0.75	2.0
norfloxacin	0.75	0.75
kanamycin	0.25	0.75

^a Σ_{FIC} , combined fractional inhibitory concentration. The formula for calculating the Σ_{FIC} is described in the Experimental Section.