

## Synergistic Effects between Conventional Antibiotics and 2-Aminoimidazole-Derived Antibiofilm Agents<sup>∇</sup>

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**2-Aminoimidazoles are an emerging class of small molecules that possess the ability to inhibit and disperse biofilms across bacterial order, class, and phylum. Herein, we report the synergistic effect between a 2-aminoimidazole/triazole conjugate and antibiotics toward dispersing preestablished biofilms, culminating with a 3-orders-of-magnitude increase of biofilm dispersion toward *Staphylococcus aureus* biofilms. Furthermore, we document that the 2-aminoimidazole/triazole conjugate will also resensitize multidrug-resistant strains of bacteria to the effects of conventional antibiotics, including methicillin-resistant *Staphylococcus aureus* (MRSA) and multidrug-resistant *Acinetobacter baumannii*.**

Bacterial biofilms and antibiotic resistance genes represent a tremendous hurdle in human health care. First, the NIH estimates that 3 in 4 bacterial infections are biofilm based (20). Bacteria within a biofilm are upwards of 1,000-fold more resistant to antibiotics and are inherently insensitive to the host immune response (20). Thus, bacteria in a biofilm represent a significant hurdle for antibiotic treatment. Second, the dissemination of antibiotic resistance genes among diverse pathogenic bacteria, coupled with the dearth of new antibiotics that have been introduced by the biomedical community, has led to a situation in which many microbial infections are multidrug resistant and extremely difficult or impossible to treat. Examples of this include the outbreaks of methicillin-resistant *Staphylococcus aureus* (MRSA) infections among healthy individuals (16) and the multidrug-resistant strains of *Acinetobacter baumannii* (MDRAB) (22) that are infecting wounded soldiers in the Middle East. In fact, more individuals die every year in the United States from complications arising from MRSA infections than from complications related to HIV (15). The lack of new strategies to combat bacterial infections has become so dire that the Infectious Diseases Society of America has recently issued a call to action for the medical community (34).

Derivatives of 2-aminoimidazoles have recently been developed as molecules that both inhibit and disperse bacterial biofilms (28). Examples of natural products in this class include oroidin, ageliferin, and mauritamine (1) (Fig. 1), and these naturally occurring secondary metabolites have been reported to have either antibiofilm or antifouling activity (14, 35, 37). Recent efforts in our lab have provided a number of synthetically accessible 2-aminoimidazole derivatives that both inhibit and disperse bacterial biofilms (3, 4, 9, 10, 11, 18, 24–27, 29, 31–33). One class of derivatives recently reported was of 2-aminoimidazole/triazole (2-AIT) conjugates (33). The most active molecule identified in this study was compound 1, and we documented that compound 1 inhibits and disperses biofilms

of *Pseudomonas aeruginosa*, *Acinetobacter baumannii*, *Bordetella bronchiseptica*, and *Staphylococcus aureus*. Growth curves and colony count assays indicated that compound 1 inhibited and dispersed bacterial biofilms without inducing cellular death.

As a therapeutic strategy, molecules that control bacterial biofilms through nonmicrobicidal mechanisms will most likely act synergistically with conventional antibiotics, cooperating advantageously to overcome an infectious threat that would otherwise persist if treated with either agent individually (36). The antibiofilm agent will maintain bacteria within their sensitive planktonic state, while the antibiotic will eliminate the bacterial population. Given this situation, it is important to determine how conventional antibiotics affect the ability of antibiofilm agents to control biofilm development and maintenance. To this end, we have studied the ability of 2-AIT compound 1 to disperse bacterial biofilms in the presence of approved antibiotics. As a follow-up study, we have investigated the ability of antibiofilm agents to affect the action of antibiotics on planktonic bacteria. Herein we report that antibiotics work synergistically with compound 1 to eliminate biofilm colonization, that compound 1 augments the action of conventional antibiotics and effectively suppresses multidrug resistance, and that compound 1 is not hemolytic at active concentrations.

### MATERIALS AND METHODS

**General experimental materials.** *A. baumannii* (ATCC 19606), *S. aureus* (ATCC 29213), *Staphylococcus epidermidis* (ATCC 29886), MRSA (ATCC BAA-44), MDRAB (ATCC BAA-1605), and a control strain of *Escherichia coli* (ATCC 35695) were obtained from the ATCC. *P. aeruginosa* strains PA14 and PDO300 were provided by Daniel J. Wozniak at the Department of Microbiology and Immunology, Wake Forest University School of Medicine. Chloramphenicol- and ampicillin-resistant *E. coli* was provided by Reza A. Ghiladi at North Carolina State University. Tetracycline-resistant *E. coli* K-12 ER2738 was purchased from New England Biolabs. Tetracycline hydrochloride (catalog number 0446012) and ampicillin sodium salt (catalog number BP1760) were purchased from Fisher Scientific. Penicillin G sodium salt (catalog number P3032), tobramycin (catalog number T4014), gentamicin sulfate salt (catalog number G4793), and erythromycin (catalog number 45673) were purchased from Sigma-Aldrich. Chloramphenicol (catalog number C0310) was purchased from Teknova. Mechanically defibrinated sheep blood (DSB100) was obtained from

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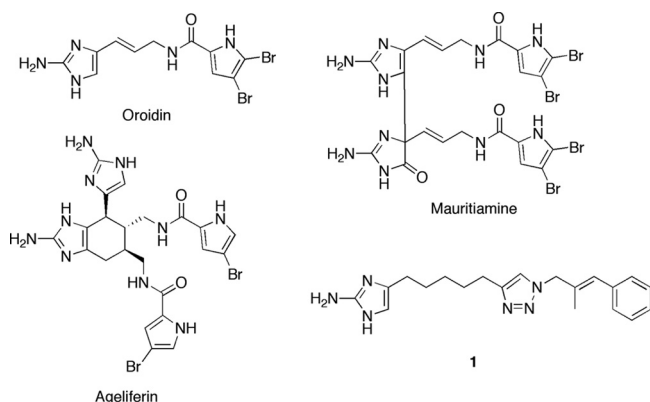


FIG. 1. 2-Aminoimidazoles that have antibiofilm and antibiofouling properties.

Hemostat Labs. MDRAB clinical isolates 3340, AB0043, and UH8407 were donated by Robert A. Bonomo. Mueller-Hinton medium was purchased from Fluka (catalog number 70192).

**Biofilm dispersion with an antibiotic or a combination of antibiotic and antibiofilm agent.** Dispersion assays were performed by taking an overnight culture of bacterial strain and subculturing it at an optical density at 600 nm ( $OD_{600}$ ) of 0.01 into the necessary medium (LB for *A. baumannii*, LB without NaCl [LBNS] for PA14 and PDO300, tryptic soy broth with a 0.5% glucose supplement [TSBG] for *S. aureus*, and tryptic soy broth with a 0.5% glucose supplement and a 3% NaCl supplement [TGN] for *S. epidermidis*). The resulting bacterial suspension was aliquoted (100  $\mu$ l) into the wells of a 96-well PVC microtiter plate. Plates were then wrapped in Glad Press'n Seal wrap, followed by an incubation under stationary conditions at ambient temperature to establish the biofilms. After 24 h, the media were discarded from the wells and the plates were washed thoroughly with water. Stock solutions of the chosen antibiotic (tobramycin for PA14 and PDO300, colistin for *A. baumannii*, and novobiocin for *S. epidermidis* and *S. aureus*) with or without compound 1 were then made in media (the antibiotic test concentration ranged initially from 0.001  $\mu$ M to 100  $\mu$ M to determine the highest concentration of antibiotic that would not disperse the preformed biofilm so it could be used in a combination study). These stock solutions were aliquoted (100  $\mu$ l) into the wells of the 96-well PVC microtiter plate with the established biofilms. Medium alone was added to a subset of the wells to serve as a control. Plates were then incubated for 24 h at 37°C. After incubation, the media were discarded from the wells and the plates were washed thoroughly with water. Plate wells were then stained with 100  $\mu$ l of a 0.1% solution of crystal violet (CV) and then incubated at ambient temperature for 30 min. Plates were washed with water again, and the remaining stain was solubilized with 200  $\mu$ l of 95% ethanol. A 125- $\mu$ l portion of the solubilized CV stain from each well was transferred to the corresponding wells of a polystyrene microtiter dish. Biofilm dispersion was quantitated by measuring the  $OD_{540}$  of each well (33).

**Quantification of the resensitization effects of compound 1 toward tetracycline-resistant *E. coli* and MRSA.** An overnight culture of the bacterial strain was subcultured to an  $OD_{600}$  of 0.01 into the necessary medium (LB for *E. coli* and TSB for MRSA). The resulting bacterial suspension was then aliquoted (3.0 ml) into culture tubes. The sensitizing agent was then added at a nonbactericidal concentration (150.0  $\mu$ M for *E. coli* and 45.0  $\mu$ M for MRSA) to the media of the test samples. Then, the choice antibiotic (tetracycline for tetracycline-resistant *E. coli* and erythromycin, gentamicin, penicillin G, and tetracycline for MRSA) was added to the test samples at nonbactericidal concentrations. Control experiments were conducted by growing bacteria with either (i) medium and sensitizing agent (no antibiotic), (ii) medium only (no antibiotic/sensitizing agent), or (iii) medium and dimethyl sulfoxide (DMSO) (amount used to administer sensitizing agent). Samples were then placed in an incubator at 37°C and shaken at 200 rpm. When the  $OD_{600}$  of the control samples reached approximately 0.4, 100  $\mu$ l was taken from each culture tube and then diluted serially into LB medium. Then, 10  $\mu$ l was removed from each serial dilution and plated out on a square-gridded petri dish, followed by 16 h of incubation at 37°C to grow viable colonies, which were quantified by the track-dilution method (6, 13).

**Broth microdilution method for MIC determination.** Overnight cultures of bacterial strain were subcultured to  $5 \times 10^5$  CFU/ml in Mueller-Hinton medium

(Fluka number 70192). The resulting bacterial suspension was aliquoted (1.0 ml) into culture tubes. Samples were prepared from these culture tubes containing either 512  $\mu$ g/ml of specified antibiotic, 512  $\mu$ g/ml of specified antibiotic with a nonbactericidal concentration of compound 1, 512  $\mu$ g/ml of compound 1, or no test compound as a control. Samples were then aliquoted (200  $\mu$ l) into the first row of wells of a 96-well microtiter plate in which subsequent wells were pre-filled with 100  $\mu$ l of a Mueller-Hinton medium-based  $5 \times 10^5$ -CFU/ml bacterial subculture (samples including compound 1 as a supplement to an antibiotic included the nonbactericidal concentration of compound 1 in this stock to keep the compound 1 concentration uniform throughout the antibiotic dilution procedure). Using the multichannel pipettor set at 100  $\mu$ l, row 1 wells were mixed 8 to 10 times. Then, 100  $\mu$ l was withdrawn and transferred to row 2. Row 2 wells were mixed 8 to 10 times, followed by a 100- $\mu$ l transfer from row 2 to row 3. This procedure was used to serially dilute the rest of the rows of the microtiter plate. The microtiter plate sample was then covered with a microtiter plate lid and placed in a covered plastic container. The chamber was incubated under stationary conditions at 37°C. After 16 h, the lid was removed and MIC values were recorded (6).

**Broth microdilution method for the determination of  $Ca^{2+}$ ,  $Mn^{2+}$ , and  $Mg^{2+}$  doping on the antibiotic resensitization activity of compound 1.** Cultures were grown in the presence or absence of 25 mg/ml  $CaCl_2$ ,  $MnCl_2$ ,  $MgSO_4$ , or 30.6 mg/ml  $CaSO_4$  for 10 to 12 h. The cultures were then subcultured to  $5 \times 10^5$  CFU/ml in Mueller-Hinton medium (Fluka number 70192) containing their respective metal ions. The resulting bacterial suspension was aliquoted (1.0 ml) into culture tubes. Samples were prepared from these culture tubes containing either 512  $\mu$ g/ml of specified antibiotic, 512  $\mu$ g/ml of specified antibiotic with a nonbactericidal concentration of compound 1, 512  $\mu$ g/ml of compound 1, or no test compound as a control (samples treated with compound 1 were allowed to stand for 30 min before the antibiotic was introduced). Samples were then aliquoted (200  $\mu$ l) into the first row of wells of a 96-well microtiter plate in which subsequent wells were pre-filled with 100  $\mu$ l of a Mueller-Hinton medium-based  $5 \times 10^5$ -CFU/ml bacterial subculture. (Samples including compound 1 as a supplement to an antibiotic included the nonbactericidal concentration of compound 1 in this stock to keep the compound 1 concentration uniform throughout the antibiotic dilution procedure, and samples supplemented with 25 mg/ml  $CaCl_2$ ,  $MnCl_2$ ,  $MgSO_4$ , or 30.6 mg/ml  $CaSO_4$  included the test concentration of these salts in the stock to keep their concentrations uniform throughout the antibiotic dilution procedure.) Using the multichannel pipettor set at 100  $\mu$ l, row 1 wells were mixed 8 to 10 times. Then, 100  $\mu$ l was withdrawn and transferred to row 2. Row 2 wells were mixed 8 to 10 times, followed by a 100- $\mu$ l transfer from row 2 to row 3. This procedure was used to serially dilute the rest of the rows of the microtiter plate. The microtiter plate sample was then covered with a microtiter plate lid and placed in a covered plastic container. The chamber was incubated under stationary conditions at 37°C. After 16 h, the lid was removed and MIC values were recorded (6).

**Microbicidal activity of compound 1 against planktonic MRSA and MDRAB.** Portions (10  $\mu$ l) were taken from individual microtiter wells from untreated MRSA and MDRAB wells and compound 1 (32  $\mu$ g/ml)-treated MRSA and MDRAB wells after the 16-hour incubation time from the previously described microdilution susceptibility testing assay. Serial dilutions were then made with the aliquots from the microtiter wells, plated out on tryptic soy agar plates, and incubated overnight. The colonies that formed were then counted and used to determine CFU  $ml^{-1}$ . At 32  $\mu$ g/ml (82.9  $\mu$ M), compound 1 did not reduce the number of viable bacterial cells of MRSA and MDRAB in comparison to the nontreated controls.

**Evolution of MRSA resistance to the effects of compound 1.** An overnight culture of the bacterial strain was subcultured to an  $OD_{600}$  of 0.01 in TSB medium. The resulting bacterial suspension was then aliquoted (3.0 ml) into culture tubes. A sublethal combination of antibiotic and compound 1 (25.0  $\mu$ M penicillin G with 45.0  $\mu$ M compound 1) was then added. Samples were then placed in an incubator at 37°C and shaken at 200 rpm for 24 h. Then, samples were subcultured at an  $OD_{600}$  of 0.01 into the necessary medium. Aliquots (3.0 ml) of these subcultures were then treated again with an identical amount of the antibiotic-compound 1 combination followed by an incubation of the samples at 37°C while being shaken at 200 rpm for 24 h. This antibiotic-compound 1 cycle was performed a total of seven times on the bacteria (within one week). After the final incubation, samples were subcultured at an  $OD_{600}$  of 0.01 into the necessary medium. The resulting bacterial suspension was then aliquoted (3.0 ml) into culture tubes. Then, an active dose (50.0  $\mu$ M penicillin G with 45.0  $\mu$ M compound 1) was administered, followed by an incubation of the samples at 37°C while being shaken at 200 rpm. Controls were employed in which no antibiotic was administered, as well as antibiotic by itself (50.0  $\mu$ M penicillin G for MRSA). When the  $OD_{600}$  of the control samples reached approximately 1.2, 100  $\mu$ l was

TABLE 1. Dispersal enhancement

Bacterial strain	EC <sub>50</sub>	Antibiotic	Antibiotic concn	Combined EC <sub>50</sub>
<i>S. epidermidis</i>	325 ± 26 nM	Novobiocin	1.0 μM	0.67 ± 0.11 nM
<i>A. baumannii</i>	121 ± 11 μM	Colistin	1.0 μM	24.8 ± 1.6 μM
<i>P. aeruginosa</i> PDO300	13.2 ± 1.1 μM	Tobramycin	10.0 μM	0.0077 ± 0.0015 μM
<i>S. aureus</i>	2.6 ± 0.6 μM	Novobiocin	0.1 μM	0.001 ± 0.00011 μM
<i>P. aeruginosa</i> PA14	22 ± 4.5 μM	Tobramycin	10.0 μM	4.72 ± 0.5 μM

taken from each culture tube and then diluted serially into the necessary medium. Then, 10 μl was removed from each serial dilution and plated out on a square-gridded petri dish, followed by 16 h of incubation at 37°C to grow viable colonies, which were quantified by the track-dilution method (13).

**Red blood cell hemolysis assay.** Hemolysis assays were performed on mechanically defibrinated sheep blood (DSB100; Hemostat Labs). Blood (1.5 ml) was placed into a microcentrifuge tube and centrifuged at 10,000 rpm for 10 min. The supernatant was removed, and then the cells were resuspended with 1 ml of phosphate-buffered saline (PBS). The suspension was centrifuged, the supernatant was removed, and cells were resuspended two more times. The final cell suspension was then diluted 10-fold. Test compound solutions were made in PBS and then added to aliquots of the 10-fold suspension dilution. PBS alone was used as a negative control and as the zero hemolysis marker, whereas a 1% Triton X sample was used as a positive control and as the 100% lysis marker. Samples were then placed in an incubator at 37°C while being shaken at 200 rpm for 1 h. After 1 h, the samples were transferred to microcentrifuge tubes and then centrifuged at 10,000 rpm for 10 min. The resulting supernatant was diluted by a factor of 40 in distilled water. The absorbance of the supernatant was measured with a UV spectrometer at a 540-nm wavelength (17).

## RESULTS AND DISCUSSION

The first question addressed was whether tandem addition of compound 1 with conventional antibiotics would show synergistic effects toward biofilm dispersion. It is known that sublethal doses of antibiotics will promote biofilm formation (8), and it was unclear if the effect of an antibiofilm agent would be mitigated under these conditions. To this end, the synergistic effect of compound 1 was studied with colistin against *A. baumannii* biofilms, with novobiocin against *S. aureus* and *S. epidermidis*, and with tobramycin against two strains of *P. aeruginosa* (PA14 and PDO300). Tobramycin was used because it is currently prescribed to cystic fibrosis patients to slow *P. aeruginosa* infections (19). Colistin was investigated because it is currently the antibiotic of last resort used for treatment of multidrug-resistant *Acinetobacter baumannii* (MDRAB) (7). Novobiocin was investigated because it has been used to treat *S. aureus* and *S. epidermidis* infections of indwelling medical devices (23).

To study synergistic effects, biofilms were first established in 96-well PVC plates for 24 h. Media and planktonic bacteria were then removed, and the wells were washed to remove any loosely adherent bacteria. Wells were then treated with either media alone, media containing antibiotic, media containing compound 1, or media containing a combination of compound 1 and the antibiotic. Biofilm mass was then quantified by using a crystal violet reporter assay (21). To quantify any synergistic effects between compound 1 and the antibiotic toward eliminating biofilm colonization, dose-response studies were performed to determine the EC<sub>50</sub> of compound 1 as a function of antibiotic concentration. This value was then compared to the EC<sub>50</sub> of compound 1 alone. Here, EC<sub>50</sub> is defined as the concentration necessary to elicit 50% dispersion of the biofilm.

As expected, the antibiotics alone did not affect biofilm mass

at all levels studied (the highest concentrations being 10 μM tobramycin for PDO300 and PA14, 1.0 μM each novobiocin and colistin for *S. epidermidis* and *A. baumannii*, respectively, and 0.1 μM novobiocin for *S. aureus*) compared to samples treated with medium only. For each bacterial strain, we observed synergistic activity between the antibiotic and compound 1 that is dependent on antibiotic concentration. These data are outlined in Table 1. As can be seen, dramatic effects are observed toward the clearance of *Staphylococcus* biofilms and, for PDO300, a mucoid variant of *P. aeruginosa* that is relevant for cystic fibrosis. For *S. aureus*, an increase in activity of 3 orders of magnitude was observed at the highest concentration of novobiocin used (EC<sub>50</sub> of compound 1 alone = 2.6 μM, EC<sub>50</sub> of compound 1 = 1.0 nM with 0.1 μM novobiocin), while for *S. epidermidis*, we observed picomolar levels of dispersion (EC<sub>50</sub> of compound 1 alone = 395 nM, EC<sub>50</sub> of compound 1 with 1.0 μM novobiocin = 670 pM). We observed moderate levels of synergy (ca. 5- to 6-fold) with the highest concentrations of antibiotics for PA14 and *A. baumannii*.

After establishing that conventional antibiotics would enhance the antibiofilm properties of compound 1, we turned to investigating the effects that compound 1 in combination with antibiotics had on the growth of multidrug-resistant strains of bacteria. There are a limited number of reports documenting that molecules that inhibit bacterial communication can suppress antibiotic resistance (38). Given that (i) the formation and dispersion of bacterial biofilms are mediated by cell-to-cell communication (5) and (ii) compound 1 inhibits and disperses bacterial biofilms from both Gram-negative and Gram-positive bacteria, we hypothesized that compound 1 may be able to resensitize antibiotic-resistant bacteria (both Gram positive and Gram negative) to the effects of antibiotics. This effect was explored using two strains of antibiotic-resistant *E. coli* (tetracycline resistant and chloramphenicol/tetracycline resistant), a MRSA clinical isolate, and multidrug-resistant *A. baumannii* (MDRAB) clinical isolates. Two tests were performed to quantify this effect. The first test was measurement of viable colonies after 4 h of growth, while the second test involved determination of MIC values.

Tetracycline-resistant *E. coli* grew identically in the absence or presence of 10 or 50 μM tetracycline. Growth of tetracycline-resistant *E. coli* in the presence of 10 or 50 μM tetracycline and 150 μM compound 1 led to a >99% reduction in bacterial growth (Fig. 2A and summarized in Table 2). In comparison, growth of an *E. coli* strain sensitive to tetracycline was reduced by only 55% with 10 μM tetracycline. We also observed >99% reduction in bacterial growth with 50 μM tetracycline and either 50 μM or 25 μM compound 1. No reduction in bacterial growth was noted when the bacterium was grown solely in the presence of 150 μM compound 1,



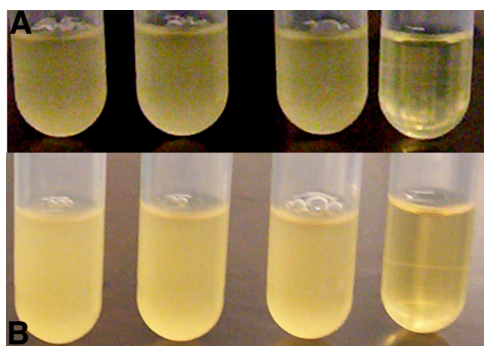


FIG. 2. (Top) Tetracycline-resistant *E. coli*. From left to right: *E. coli* control, *E. coli* with 50  $\mu$ M tetracycline, *E. coli* with 150  $\mu$ M compound 1, *E. coli* with 50  $\mu$ M tetracycline and 150  $\mu$ M compound 1. (Bottom) MRSA strain BAA-44. From left to right: MRSA control, MRSA with 25  $\mu$ M methicillin, MRSA with 45  $\mu$ M compound 1, MRSA with 25  $\mu$ M methicillin and 45  $\mu$ M compound 1.

confirming the absence of microbicidal activity for compound 1 and synergistic activity between compound 1 and the antibiotic.

Next, we investigated the ability of compound 1 to resensitize a multidrug-resistant strain of MRSA (BAA-44). BAA-44 grew identically in the absence or presence of the following antibiotics: (i) 150  $\mu$ M gentamicin, (ii) 100  $\mu$ M erythromycin, (iii) 25  $\mu$ M penicillin G, (iv) 25  $\mu$ M methicillin, and (v) 1  $\mu$ M tetracycline. BAA-44 grew identically in the presence or absence of 45  $\mu$ M compound 1 (Table 2). However, combining each antibiotic individually with bacteria preexposed to compound 1 caused a dramatic reduction in bacterial growth in each case. The combination of 150  $\mu$ M gentamicin–45  $\mu$ M compound 1 caused 86% reduction in BAA-44 growth, while 100  $\mu$ M erythromycin–45  $\mu$ M compound 1 caused 82% reduction in BAA-44 growth. In comparison, growth of antibiotic-sensitive *S. aureus* is reduced by >99% or 89% in the presence of 150  $\mu$ M gentamicin or 100  $\mu$ M erythromycin, respectively. In the presence of 45  $\mu$ M compound 1 and either 25  $\mu$ M penicillin G, 25  $\mu$ M methicillin, or 1  $\mu$ M tetracycline, BAA-44 growth was reduced 95%, 91%, and 69%, respectively (Fig. 2B). Growth of antibiotic-sensitive *S. aureus* was reduced by 91%, >99%, and 45% in the presence of 25  $\mu$ M penicillin G, 25  $\mu$ M methicillin, and 1  $\mu$ M tetracycline, respectively.

TABLE 3. MIC measurement of antibiotic sensitization

Organism and antibiotic	MIC $\mu$ g/ml of:		
	Drug alone	Drug with 45 $\mu$ M <sup>a</sup> of compound 1	Drug with 75 $\mu$ M <sup>a</sup> of compound 1
<b>MRSA</b>			
Penicillin G	32	4	
Tetracycline	16	16	
Methicillin	256	64	
Ciprofloxacin	4	2	
Compound 1	64		
<b>MDRAB</b>			
Imipenem	16		2
Ciprofloxacin	64		16
MDRAB 3340, imipenem	64		8
MDRAB AB0043, imipenem	16		4
MDRAB UH8407, imipenem	1		0.125

<sup>a</sup> Compound 1 at 32  $\mu$ g/ml (83  $\mu$ M) was determined to be nontoxic to planktonic MRSA and MDRAB via colony count analysis.

After establishing that a combination of compound 1 with conventional antibiotics would affect bacterial growth, we established the MIC values of each antibiotic in the absence or pretreated presence of compound 1 against all of the aforementioned bacterial strains. The MIC measurement differs from the enumeration of viable bacteria used above in two key aspects. The first is the time frame of the assay (4 h versus 16 to 24 h), and the second is the stringency of the assay. The bacterial growth assay is a measurement of how the bacteria respond under given conditions, while the MIC assay reports the concentration at which a given treatment causes no visible bacterial growth and is the accepted standard for defining the *in vitro* efficacy of a potential treatment approach.

MIC values for each bacterial strain were first established for compound 1 and then for each antibiotic separately studied using the microdilution protocol (6). These results are summarized in Table 3. The MIC values determined with each antibiotic against each strain were in agreement with established MIC values (6). The MIC value of compound 1 against the MRSA strain was determined to be 64  $\mu$ g/ml, while the MIC value of compound 1 against the *A. baumannii* strains was determined to be 64  $\mu$ g/ml. MIC values of each antibiotic were

TABLE 2. Colony count measurement of antibiotic sensitization

Bacterial strain	Antibiotic	Concn of antibiotic ( $\mu$ M)	Growth reduction (%)	Concn of compound 1 ( $\mu$ M) added	Growth reduction (%) with antibiotic and compound 1
MRSA	Gentamicin	150.0		45.0	86.3 $\pm$ 0.4
MRSA	Erythromycin	100.0		45.0	81.9 $\pm$ 2.3
MRSA	Penicillin	25.0		45.0	94.8 $\pm$ 1.0
MRSA	Methicillin	25.0		45.0	90.5 $\pm$ 1.7
MRSA	Tetracycline	1.0		45.0	69.2 $\pm$ 2.3
<i>S. aureus</i>	Gentamicin	150	99.8 $\pm$ 0.1		
<i>S. aureus</i>	Erythromycin	100.0	89.3 $\pm$ 0.9		
<i>S. aureus</i>	Penicillin	25.0	90.6 $\pm$ 2.7		
<i>S. aureus</i>	Methicillin	25.0	99.9 $\pm$ 0.1		
<i>E. coli</i> (Tet <sup>r</sup> )	Tetracycline	50.0		150.0	99.9 $\pm$ 0.01
<i>E. coli</i> (Tet <sup>r</sup> )	Tetracycline	10.0		150.0	99.9 $\pm$ 0.1
<i>E. coli</i>	Tetracycline	10.0	55.3 $\pm$ 3.7		

then determined in the presence of compound 1. For the *A. baumannii* strains we tested 75  $\mu\text{M}$  compound 1, while we tested 45  $\mu\text{M}$  compound 1 for MRSA. Against both *A. baumannii* and MRSA, 82.5  $\mu\text{M}$  compound 1 demonstrated no bactericidal activity. By screening at concentrations of compound 1 that did not elicit any measurable effect on bacterial growth, any reduction in MIC values could be attributed to resensitization activity. MRSA and MDRAB were chosen specifically for MIC studies because they are primary clinical isolates and thus are representative pathogens encountered in a hospital setting.

The results of these experiments for MRSA are summarized in Table 3. An 8-fold (32  $\mu\text{g}/\text{ml}$  to 4  $\mu\text{g}/\text{ml}$ ) drop and a 4-fold (256  $\mu\text{g}/\text{ml}$  to 64  $\mu\text{g}/\text{ml}$ ) drop in MIC were noted with penicillin G and methicillin, respectively, in combination when the bacteria were pretreated with 45  $\mu\text{M}$  compound 1. In comparison, the MIC values for  $\beta$ -lactam-sensitive *S. aureus* were 2  $\mu\text{g}/\text{ml}$  with methicillin and 1  $\mu\text{g}/\text{ml}$  with penicillin G. This effect was not noted (where we define the difference in MIC value to be 2-fold or less) for tetracycline, with which the MIC remained at 16  $\mu\text{g}/\text{ml}$ , or for ciprofloxacin, with which the MIC was reduced only from 4  $\mu\text{g}/\text{ml}$  to 2  $\mu\text{g}/\text{ml}$ .

For the MDRAB strain obtained from the ATCC (BAA-1605), the MIC of imipenem was reduced from 16  $\mu\text{g}/\text{ml}$  to 2  $\mu\text{g}/\text{ml}$  after pretreatment of the bacteria with 75  $\mu\text{M}$  compound 1. A similar effect was noted for ciprofloxacin, with which the MIC was reduced from 64  $\mu\text{g}/\text{ml}$  to 16  $\mu\text{g}/\text{ml}$  after pretreatment with 75  $\mu\text{M}$  compound 1. For antibiotic-sensitive *A. baumannii*, the MICs were determined as 0.25  $\mu\text{g}/\text{ml}$  (imipenem) and 0.5  $\mu\text{g}/\text{ml}$  (ciprofloxacin).

Three additional MDRAB clinical isolate strains (AB0043, UH8407, and 3340) were then tested for resensitization effects. The most well-characterized clinical specimen in this study was AB0043, an isolate from a patient suffering from an *A. baumannii* infection at Walter Reed Army Medical Center. This bacterial strain was isolated in 2004 from the blood of a soldier that was deployed to Iraq/Kuwait and was found to be resistant to seven of the nine antibiotics used in the study to determine its MDR phenotype. Genetic analysis showed that this isolate demonstrated enhanced efflux pump activity via *adeR* expression, which typically makes isolates resistant to aminoglycosides, quinolones, tetracycline, and trimethoprim. Several aminoglycoside-modifying enzymes (AMEs) were expressed in this clinical isolate as well. Isolate AB0043 was found to express the AME gene *aadB*, which was often associated with tobramycin resistance of *A. baumannii* clinical isolates (12).

For MDRAB clinical isolate 3340 (Table 3), the MIC values of imipenem were reduced after a pretreatment with 75  $\mu\text{M}$  compound 1 (64  $\mu\text{g}/\text{ml}$  to 8  $\mu\text{g}/\text{ml}$ ), while similar effects were also noticed for strain AB0043, with which the MIC values of imipenem dropped after a pretreatment with 75  $\mu\text{M}$  compound 1 (16  $\mu\text{g}/\text{ml}$  to 4  $\mu\text{g}/\text{ml}$ ). Lastly, for clinical isolate UH8407, the MIC values of imipenem also dropped after a pretreatment with 75  $\mu\text{M}$  compound 1 (1  $\mu\text{g}/\text{ml}$  to 0.125  $\mu\text{g}/\text{ml}$ ). No significant reduction (>2-fold) in MICs of ciprofloxacin was observed.

We also wanted to investigate if the resensitization effect was due directly to maintaining bacteria in their planktonic state (i.e., biofilm inhibition or dispersion) or if it was due to a specific mechanism attributable to compound 1. To address

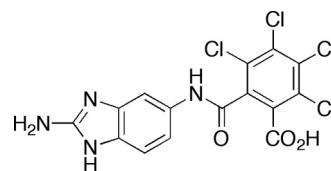


FIG. 3. A 2-aminobenzimidazole that inhibits and disperses Gram-positive biofilms through a zinc-dependent mechanism.

this question, we have recently developed a 2-aminobenzimidazole (2-ABI) (Fig. 3) that inhibits and disperses Gram-positive biofilms (MRSA, *S. epidermidis*, and vancomycin-resistant *Enterococcus faecium*) at low micromolar/high nanomolar concentrations through a zinc-dependent mechanism (31). This 2-ABI conjugate was screened at 45  $\mu\text{M}$  in the presence of 25  $\mu\text{M}$  methicillin and was unable to elicit a resensitizing response against MRSA. Therefore, resensitization activity appears to be attributable to a specific mechanism associated with compound 1, not a generic phenomenon of simply inhibiting/dispersing biofilms.

We also noticed a time dependence of exposure to compound 1 to the sensitization activity efficacy before the introduction of the antibiotic. This effect was delineated by determining the MIC values of penicillin G against MRSA with both a 30-min and a 60-min exposure to 45  $\mu\text{M}$  compound 1 prior to antibiotic addition. The penicillin G MIC of 45  $\mu\text{M}$  compound 1 with a 30-min exposure was found to be 2  $\mu\text{g}/\text{ml}$ , whereas that with a 60-min exposure was 0.25  $\mu\text{g}/\text{ml}$ .

Once we had established the ability of compound 1 to resensitize MDR bacteria to the effects of conventional antibiotics, we performed preliminary mechanism of action studies. We noting that switching from standard Mueller-Hinton broth (MHB) prepared in our lab (stock solid purchased from Fluka) to preprepared cation-adjusted Mueller-Hinton broth (CAMHB) purchased from Becton Dickinson significantly suppressed the resensitization activity of compound 1. The difference between the two media is that CAMHB has been supplemented with calcium(II) and magnesium(II) ions to yield media with final concentrations of 20 to 25 mg/liter of Ca(II) and 10 to 12.5 mg/liter of Mg(II), respectively. Therefore, we initially supplemented our MHB with both  $\text{CaCl}_2$  (an additional 25 mg/liter) and  $\text{MgSO}_4$  (an additional 25 mg/liter) and evaluated the ability of compound 1 to resensitize MRSA to the effects of penicillin G. In these new media, at 45  $\mu\text{M}$  compound 1, we observed only a modest 2-fold reduction in the MIC of penicillin G (32  $\mu\text{g}/\text{ml}$  to 16  $\mu\text{g}/\text{ml}$ ), indicating that metal cations can suppress the resensitization effects. We then individually supplemented our MHB with either  $\text{CaCl}_2$  (an additional 25 mg/liter) or  $\text{MgSO}_4$  (an additional 25 mg/liter). Only the MHB supplemented with  $\text{CaCl}_2$  suppressed the resensitization activity of compound 1.

Once we had determined that calcium(II) levels modulate the resensitization activity of compound 1, we set up a number of experiments to quantify these effects (Table 4). We first set up resensitization experiments with  $\text{CaSO}_4$  and penicillin G, MRSA, and compound 1 to determine if the effect was due to Ca(II) or the chloride counterion.  $\text{CaSO}_4$  modulated the activity of compound 1 in a fashion similar to that of  $\text{CaCl}_2$ , indicating that Ca(II) was responsible for suppressing the re-

TABLE 4. Metal doping effects on the antibiotic resensitization ability of compound 1

Organism and antibiotic	MIC ( $\mu\text{g/ml}$ ) <sup>a</sup> with:				
	Mueller-Hinton	CaSO <sub>4</sub>	CaCl <sub>2</sub>	MgSO <sub>4</sub>	MnCl <sub>2</sub>
<b>MRSA</b>					
Penicillin G	32	32	32	32	32
Penicillin G with 45 $\mu\text{M}$ compound 1	4	16	16	4	16
Penicillin G with 90 $\mu\text{M}$ compound 1	0.125	2	2	0.125	8
<b>MDRAB</b>					
Imipenem	16		16	16	16
Imipenem with 75 $\mu\text{M}$ compound 1	2		8	2	8
Imipenem with 150 $\mu\text{M}$ compound 1	0.125		4	0.125	2

<sup>a</sup> For doping, 25  $\mu\text{g/ml}$  of CaCl<sub>2</sub>, MgSO<sub>4</sub>, or MnCl<sub>2</sub> or 30.7  $\mu\text{g/ml}$  of CaSO<sub>4</sub> was used.

sensitization activity of compound 1. For example, in MHB supplemented with 30.7 mg/liter CaSO<sub>4</sub> and 45  $\mu\text{M}$  compound 1, the MIC of penicillin G against MRSA was 16  $\mu\text{g/ml}$ . In comparison, the MIC of penicillin G against MRSA was 32  $\mu\text{g/ml}$  in MHB and 4  $\mu\text{g/ml}$  in MHB with 45  $\mu\text{M}$  compound 1. We also reverified that the activity was due to excess calcium by determining the MIC of penicillin G against MRSA in MHB supplemented with 225  $\mu\text{M}$  CaCl<sub>2</sub> (25 mg/liter), 225  $\mu\text{M}$  EGTA, and 45  $\mu\text{M}$  compound 1. EGTA is a calcium(II)-specific chelator that will sequester any available Ca(II); thus, if free calcium cations are suppressing the activity of compound 1, the addition of EGTA should allow compound 1 to resensitize MRSA to the effects of antibiotics. The MIC of penicillin G in this medium (MHB, CaCl<sub>2</sub>, EGTA, compound 1) was 4  $\mu\text{g/ml}$ , identical to the MIC determined in MHB supplemented with 45  $\mu\text{M}$  compound 1. As a control, we determined that the MIC of penicillin G in MHB with 225  $\mu\text{M}$  EGTA was 32  $\mu\text{g/ml}$ , which is identical to the MIC in MHB only.

In terms of bioactivity, manganese(II) can typically be used as a surrogate for calcium(II) when studying calcium-dependent processes (2). Therefore, we elected to investigate if manganese(II) would also attenuate the activity of compound 1. The MIC of penicillin G against MRSA in MHB supplemented with 25 mg/liter MnCl<sub>2</sub> and 45  $\mu\text{M}$  compound 1 was calculated as 16  $\mu\text{g/ml}$ , identical to the result for MHB with CaCl<sub>2</sub> and compound 1.

After delineating this metal-dependent activity with MRSA, we explored whether these effects also applied to MDRAB. We performed parallel experiments with one of the *A. baumannii* isolates (BAA-1605) and observed that the resensitization activity of 75  $\mu\text{M}$  compound 1 was significantly muted in MHB supplemented with an additional 25 mg/liter CaCl<sub>2</sub> (8  $\mu\text{g/ml}$  for imipenem) versus that in unsupplemented MHB (2  $\mu\text{g/ml}$  for imipenem). In comparison, the MIC of imipenem against this MDRAB strain in MHB was 32  $\mu\text{g/ml}$ . As with the MRSA strain, Mn(II) also suppressed the ability of compound 1 to resensitize the MDRAB strain to antibiotics. We also noted that increasing the concentration of compound 1 offset the effects of additional Ca(II) for both the MRSA and the MDRAB clone (Table 4), thus indicating that further analogue design to optimize activity can be employed if the activity of

compound 1 is significantly impaired *in vivo* by excess calcium ions.

Calcium(II) is also known to have a significant effect on bacterial biofilm formation (2). Therefore, we investigated whether the addition of Ca(II) would mitigate the antibiofilm effects of compound 1 and perhaps indicate that a single bacterial target can be modulated to control both biofilm formation and certain classes of antibiotic resistance (Fig. 4). BAA-44 biofilm formation was completely inhibited by 100  $\mu\text{M}$  compound 1 in tryptic soy broth with a 0.5% glucose supplement (TSBG), while in TSBG supplemented with either 25 mg/liter CaCl<sub>2</sub> or 25 mg/liter MnCl<sub>2</sub>, 100  $\mu\text{M}$  compound 1 inhibited only 40% or 27% of MRSA biofilm formation. Control experiments of biofilms grown with TSBG medium supplemented with either 25 mg/liter CaCl<sub>2</sub> or 25 mg/liter MnCl<sub>2</sub> showed biofilm masses identical to those of MRSA grown in TSBG only.

With any given antibacterial strategy, evolution of resistance is always a significant problem. Therefore, we evaluated the ability of MRSA clone BAA-44 to evolve resistance to compound 1. BAA-44 was inoculated into 3 ml of media containing 45  $\mu\text{M}$  compound 1 and 25  $\mu\text{M}$  penicillin G. Bacteria were allowed to grow for 24 h, at which time 100  $\mu\text{l}$  of the bacterial solution was used to inoculate another 3 ml of medium containing 45  $\mu\text{M}$  compound 1 and 25  $\mu\text{M}$  penicillin G. This process was repeated for 1 week. Four-hour growth analysis of the resulting bacterial culture was then monitored in either (i) medium only, (ii) medium containing compound 1, (iii) medium containing penicillin G, (iv) medium containing methicillin, (v) medium containing compound 1 and penicillin G, or (vi) medium containing compound 1 and methicillin. Growth characteristics of the resulting bacterial culture were identical to those of growth previously determined (Table 2). Therefore, selection pressures to develop compound 1 resistance are either fully or significantly repressed.

Finally, red blood cell hemolysis analysis (17) of compound 1 was also performed using defibrinated sheep blood. The HD<sub>50</sub> (hemolytic dose that lyses 50% of the red blood cells) was found to be 800  $\mu\text{M}$ . Even more promising is the fact that hemolysis was not detected until after compound 1 reached 400  $\mu\text{M}$ , a concentration that is well above the concentration of compound 1 used to elicit the sensitization response.

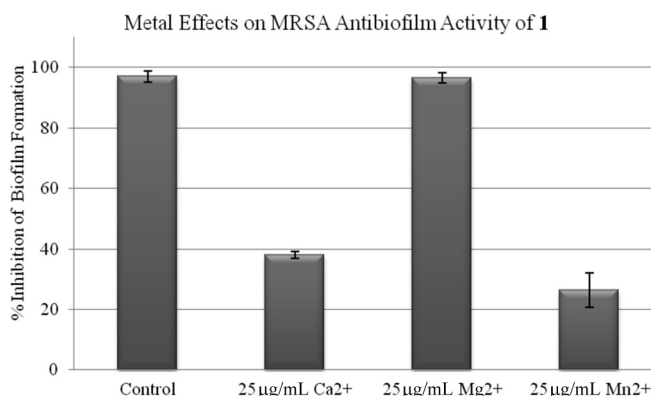


FIG. 4. Metal effects on the ability of compound 1 to inhibit MRSA biofilm formation.



In conclusion, we have demonstrated that the combination of an antibiofilm agent containing a 2-aminoimidazole/triazole motif with conventional antibiotics provides an effective strategy for remediating biofilm colonization. Furthermore, we have shown that it is possible to employ the 2-aminoimidazole/triazole conjugate compound 1 as an adjuvant for resensitizing multidrug-resistant bacteria to the effects of conventional antibiotics. Currently, we are further deconvoluting the mechanism of action of compound 1 as it applied to both its antibiofilm activity and its resensitization activity to potentially provide novel therapeutic strategies/targets for the treatment of bacterial infections. Given that 2-aminoimidazoles have been shown to be nontoxic in both cell culture and model organism models of toxicity (9, 30), we are also tuning the molecular architecture of compound 1 to enhance its resensitization activity to evaluate the *in vivo* potential of 2-aminoimidazoles to serve as adjuvants for the treatment of multidrug-resistant bacterial infections.

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