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A 2-Aminobenzimidazole That Inhibits and Disperses Gram-Positive Biofilms through a Zinc-Dependent Mechanism

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Bacterial biofilms, which are defined as surface-attached communities of bacteria encased in an extracellular matrix of biomolecules, represent a significant hurdle for infectious disease control.¹ Within the biofilm state, bacteria are upward of 1000-fold more resistant to antibiotics and are inherently resistant to host immune responses.² The National Institutes of Health estimate that 75% of all infections are biofilm-based and that biofilms drive many hospital-acquired infections (such as those resulting from implanted medical devices).³

Because of the prominence of biofilms in the biomedical community, there has been a significant effort to identify molecules that target bacteria in the biofilm state.⁴ To define additional motifs with antibiofilm properties, we became interested in assessing the activity of 2-aminobenzimidazole (2-ABI) derivatives. The decision to study the 2-ABI scaffold was based upon previous studies in our group that analyzed the antibiofilm properties of a number of small molecules based upon the natural product bromoageliferin (Figure 1).^{5–7} One of the first derivatives studied was TAGE,⁶ a bicyclic 2-aminoimidazole (2-AI) that represents the core architecture of bromoageliferin. The 2-ABI scaffold is a readily accessible, aromatized analogue of TAGE, which we hypothesized would provide unique and/or improved antibiofilm properties in comparison with 2-AIs.



Figure 1

To explore this hypothesis, a preliminary library of 2-ABI analogues was synthesized for antibiofilm evaluation (Scheme 1). An isomeric mixture of tri-Boc-protected 5-amino-2-ABI $(1)^8$ was acylated with an array of cyclic anhydrides, and the products were subsequently deprotected to generate an initial set of isomerically pure 2-ABI derivatives. A related set of 2-ABI derivatives was also synthesized in which reaction of 1 with cyclic anhydrides under refluxing toluene followed by Boc deprotection delivered the target 2-ABI-imides.

Each compound was screened at 100 μ M for its ability to inhibit the formation of *Pseudomonas aeruginosa* PAO1 and multidrugresistant *Acinetobacter baumannii* (MDRAB) biofilms. As opposed to the 2-AI class of antibiofilm agents, none of the 2-ABI derivatives were able to inhibit formation of biofilms of either γ -proteobacteria. Next, each compound was screened for its ability to inhibit biofilm





 a Reaction conditions: (a) cyclic anhydride, CH_2Cl_2; (b) HCl, H_2O, THF; (c) cyclic anhydride, toluene, 110 °C.

development in three Gram-positive bacterial strains that are prominent in human medicine. The three strains chosen were MRSA, vancomycin-resistant Enterococcus faecium (VRE), and Staphylococcus epidermidis. Again, all of the 2-ABI derivatives were initially screened at 100 μ M. Each compound was able to inhibit biofilm development of at least two of the bacterial strains. A dose-response study was then initiated to determine both the IC₅₀ and EC₅₀ values of each compound toward the bacterial strains (Table 1). Here, IC₅₀ is defined as the concentration of compound that inhibits biofilm development by 50%, while EC_{50} is defined as the concentration of compound that disperses 50% of a preformed biofilm. Of these compounds, 2-ABI derivative 3 had the best activity profile, with IC₅₀ and EC₅₀ values of 890 nM and 2.9 μ M (MRSA), 1.4 μ M and 75 μ M (VRE), and 570 nM and 7.3 μ M (S. epidermidis), respectively. Growth-curve and colony-count analyses demonstrated that each compound was nonmicrobicidal at the IC₅₀ value

Next, the mechanistic basis of how **3** was able to inhibit and disperse bacterial biofilms was investigated. Iron levels are known to effect Gram-positive biofilm development⁹ and were deemed a plausible driver of the observed antibiofilm activity. To examine Fe(II)-related antibiofilm behavior, a dose-dependent study was performed against each Gram-positive bacteria in which the ability of **3** to inhibit biofilm development was measured under increasing Fe(II) concentration. It was noted that the activity of **3** was not affected by increasing the Fe(II) concentration, thus indicating iron homeostasis is not involved in 2-ABI antibiofilm activity.

Next, Zn(II) homeostasis was examined. Zn(II) has been implicated in the pathogenesis of Gram-positive bacterial infections

Table 1. Biofilm Inhibition and Dispersion Data

	IC ₅₀ (μM); EC ₅₀ (μM)		
compound	MRSA	VRE	S. epidermidis
2 3 4	$\begin{array}{c} 5.9 \pm 1.3; 35 \pm 2.8 \\ 0.89 \pm 0.01; 2.9 \pm 0.4 \\ 1.5 \pm 0.4; 6.6 \pm 0.2 \end{array}$	$\begin{array}{c} 21 \pm 2.9; 75 \pm 6.7 \\ 1.4 \pm 0.4; 75 \pm 2.1 \\ 210 \pm 35; 280 \pm 8.3 \end{array}$	$\begin{array}{c} 32 \pm 3.8; 44 \pm 7.9 \\ 0.57 \pm 0.2; 7.3 \pm 0.2 \\ 1.9 \pm 0.4; 19 \pm 5.8 \end{array}$
5 6 7	$16 \pm 2.9; 63 \pm 6.9$ >300; >300 $69 \pm 2.7; 205 \pm 15$	$\begin{array}{c} 63 \pm 9.1; 107 \pm 11 \\ 22 \pm 8.3; 88 \pm 5.2 \\ 0.9 \pm 0.3; 94 \pm 10 \end{array}$	$\begin{array}{l} 4.1 \pm 2.0; 45 \pm 6.1 \\ 1.9 \pm 0.9; 13 \pm 6.1 \\ 240 \pm 20; 260 \pm 6.7 \end{array}$
8 9 10	$103 \pm 6.3; 250 \pm 19$ >300; >300 $2.8 \pm 0.8; 36 \pm 1.7$	$\begin{array}{c} 2.4 \pm 0.7; > 300 \\ 0.9 \pm 0.4; 6.3 \pm 1.9 \\ 7.7 \pm 0.8; 150 \pm 8.4 \end{array}$	$\begin{array}{c} 73 \pm 8.2; 175 \pm 8.5 \\ 6.2 \pm 1.2; 30 \pm 5.7 \\ 180 \pm 17; 266 \pm 7.3 \end{array}$
11 12 13	$\begin{array}{c} 17 \pm 1.9; 25 \pm 2.0 \\ 48 \pm 4; 101 \pm 6 \\ 9.6 \pm 0.8; 14 \pm 2.9 \end{array}$	$\begin{array}{c} 1.6 \pm 0.9; 280 \pm 4.8 \\ 16 \pm 4.7; 203 \pm 5.5 \\ 6.9 \pm 2.2; 30 \pm 8.5 \end{array}$	$12 \pm 1.0; 137 \pm 15 \\ 0.9 \pm 0.3; 17 \pm 4.0 \\ 15 \pm 2.7; 70 \pm 4.2$
14 15 16	$\begin{array}{l} 42 \pm 2.4; 48 \pm 2.1 \\ > 300; > 300 \\ 5.7 \pm 0.5; 9.5 \pm 2.5 \end{array}$	$\begin{array}{c} 1.9 \pm 0.5; 230 \pm 9.6 \\ 69 \pm 10; 110 \pm 2.9 \\ 7.4 \pm 1.7; 31 \pm 7.8 \end{array}$	$\begin{array}{c} 170 \pm 14; 180 \pm 13 \\ 24 \pm 6.3; 81 \pm 4.9 \\ 1.5 \pm 0.5; 33 \pm 3.7 \end{array}$

and is an important regulator of biofilm formation.¹⁰ Furthermore, generic Zn(II) chelators such as diethylenetriaminepentaacetic acid (DTPA) are known to inhibit the formation of *Staphylococcus* spp. biofilms at midmicromolar concentrations.¹⁰ As opposed to the Fe(II) study, it was noted that Zn(II), in a dose-dependent manner against each Gram-positive bacteria, suppressed the ability of **3** to inhibit biofilm development. When supplemented with 200 μ M ZnCl₂, **3** was unable to inhibit biofilm formation.

In view of this Zn(II) dependence, the ability of **3** to bind zinc directly was examined to ascertain whether the biofilm inhibition was potentially occurring via a Zn(II)-binding mechanism. To answer this question, an ¹H NMR binding experiment was performed in which the chemical shifts of **3** were measured in the presence of 0, 0.5, and 1.0 equiv of ZnCl₂ (Figure 2). Comparison of the aromatic peaks clearly indicates peak broadening with increasing amounts of ZnCl₂, indicating that **3** directly binds ZnCl₂. As a control, the same experiment was performed with FeSO₄. No change in the NMR signal of **3** was observed with 0, 0.5, or 1.0 equiv of FeSO₄.



Figure 2. NMR spectra of 228 mM 3 with (A) 0.0, (B) 0.5, and (C) 1.0 equiv of $ZnCl_2$.

Finally, two control compounds were tested (**17** and **18**; Figure 3) to probe whether substructures within **3** were responsible for

the antibiofilm properties of the compound. Neither compound was able to inhibit MRSA, VRE, or *S. epidermidis* biofilm formation at 100 μ M (the highest concentration tested). Furthermore, no change was noted in either ¹H NMR spectrum in the presence of ZnCl₂.



Figure 3. Control compounds.

In conclusion, a novel inhibitor and disperser of Gram-positive biofilms that is based upon a 2-ABI scaffold and operates via a Zn(II)-dependent mechanism has been identified. Preliminary NMR studies indicated that this compound binds zinc directly. These 2-ABI molecules are unique in that they are some of the most potent antibiofilm agents identified to date that do not operate through a microbicidal mechanism. Furthermore, molecules that bind Zn(II) have recently been shown to be efficacious in animal models of disease;^{11,12} therefore, appropriately designed 2-ABI molecules may provide a basis for remediating biofilm-based infections.

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Supporting Information Available: Experimental procedures and characterization data for all new compounds; planktonic growth curves for MRSA, VRE, and *S. epidermidis* in the presence and absence of active 2-ABIs; dose—response curves of **3** in the absence and presence ZnCl₂ and FeSO₄; and dose—response curves and NMR binding studies for **17** and **18**. This material is available free of charge via the Internet at http://pubs.acs.org.

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