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Identification of BfmR, a Response Regulator Involved in Biofilm Development, as a Target for a 2-Aminoimidazole-Based Anti-Biofilm Agent

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Abstract

2-aminoimidazoles (2AIs) have been documented to disrupt bacterial protection mechanisms, including biofilm formation and genetically-encoded antibiotic resistance traits. Using *Acinetobacter baumannii*, we provide initial insight into the mechanism of action of a 2AI-based antibiofilm agent. Confocal microscopy confirmed that the 2AI is cell permeable, while pull-down assays identified BfmR, a response regulator that is the master controller of biofilm formation, as a target for this compound. Binding assays demonstrated specificity of the 2AI for response regulators, while computational docking provided models for 2AI/BfmR interactions. The 2AI compound studied here represents a unique small molecule scaffold that targets bacterial response regulators.

Acinetobacter baumannii is a Gram-negative opportunistic human pathogen that causes nosocomial infections in immunocompromised patients[1]. The problem is compounded because *A. baumannii* survives for long periods by forming biofilms on surfaces and medical devices[2,3]. Persisting in all environments as a biofilm allows the bacterium enhanced opportunity to enter the body via open wounds, catheters and breathing tubes causing infections from pneumonia to septicemia[4].

We have found that simple derivatives of the natural products oroidin and bromoageliferin inhibit and disperse biofilms from both Gram-negative and Gram-positive bacteria,[5–10] fungi[11] and mixed species biofilms[11]. Furthermore, we have also demonstrated that these 2-aminoimidazole (2AI) compounds work synergistically with conventional antibiotics to eradicate bacteria within a biofilm state as well as resensitize multidrug resistant bacteria to

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ASSOCIATED CONTENT

Supporting Information

Materials and methods for confocal microscopy, pull-down experiments and docking. Figures for 2AI-1 analogs and fluorescence experiments. Sequence alignments for response regulators. This material is available free of charge via the Internet at http://pubs.acs.org.

Author Contributions

RJT, BGB, SDS, ALO, LP, ZS, LAA, CM, JC performed and analyzed the experimental work. JC, CM wrote the manuscript with contributions from all authors.

Notes

The authors declare no competing financial interest.

the effects of numerous antibiotics while the bacteria are in their planktonic state[12]. At this time mechanistic information to describe how these 2AI compounds control bacterial behavior has remained elusive. In this work we show that one of our 2AI compounds targets the response regulator protein BfmR from *A. baumannii*. BfmR has been shown to be a master controller of biofilm formation in *A. baumannii*[13].

Response regulators constitute one half of the bacterial communication module known as a two-component system. Two-component systems enable bacteria to sense and respond to changes in environmental conditions. They characteristically consist of a membrane-bound histidine kinase that senses a specific environmental stimulus and a matching response regulator that mediates the bacterial response, typically through differential expression of target genes[14]. In addition to providing environmental surveillance, two-component systems are essential elements of the virulence and antibiotic resistance responses of opportunistic bacterial pathogens[15,16]. Not surprisingly they have long been considered an ideal therapeutic target in the infectious disease community. So far, nearly all therapeutic efforts have focused on affecting the histidine kinases[17–20]. A recent virtual screening approach was used to design molecules that affect the response regulator PhoP binding DNA, but no *in vivo* or clinical studies have been reported[21].

The 2AI compound used in this study (2AI-1) is shown in Fig. 1a. This molecule has demonstrated great efficacy in affecting *A. baumannii* in both its biofilm and planktonic states[12,22.23].

Our first goal was to determine whether 2AI-1 was able to cross both *A. baumannii* cell membranes and enter the cytoplasm. To do this we used a fluorescein-labeled analog of 2AI-1 and confocal laser scanning microscopy or CLSM (Supporting Information). Prior to applying the 2AI-1 analog, we used 'impermeable' octadecyl-rhodamine B (ORB) to establish a negative control[24]. The ORB fluorophore binds to the environmentally exposed surface of the outer membrane and cannot cross into the cell. When viewed using CLSM, the ORB-treated cells exhibit a red halo that outlines the cells surface (Fig. 1b). After establishing this control, *A. baumannii* bacteria were treated with 100 μM of the 2AI-1 fluorescein analog, and live samples were viewed after a 30-minute incubation. The fluorescein signal in the live *A. baumannii* sample was uniformly distributed throughout the cells with the appearance of hotspots just within the cellular boundary, confirming that 2AI-1 was able to permeate the membrane barriers (Fig. 1c). In addition to live samples, formaldehyde-fixed samples were made from the analog treated cultures. These samples revealed that the analog could be crosslinked, due to the free amine moiety on the 2AI ring, within the cellular membranes and internal hotspots, especially near septa (Fig. 1d), supporting the membrane permeability of the molecule.

Next we determined a biological target for 2AI-1. Unlike for typical antibiotics, 2AI mechanistic studies are hampered by the fact that the compounds exert their biological control through a non-microbicidal mechanism. Consequently, there is no underlying evolutionary basis allowing the bacteria to evolve resistance traits and hence to deliver mutants to be sequenced. Indeed, we have attempted to employ antibiotics as adjuvants to induce bacterial stress and generate mutants that are resistant to the effects of our 2AI derivatives. To date this approach has proved fruitless[11]. Given this restraint, we elected to identify potential protein targets directly using a pull-down strategy in conjunction with mass spectrometry. In these studies we employed *A. baumannii* bacterial lysate and a functionally active biotinylated analog of 2AI-1. The compound 2AI-1 falls under our “reverse amide” class of anti-biofilm agents[22,25] and we have previously shown that the tertiary amide analogue 2AI-2 is an active anti-biofilm agent against *A. baumannii*[23]. In addition, further functionalization of the alkyne via click chemistry does not erode activity.

Therefore, we chose to pursue conjugation to biotin via the alkyne linker. The resulting biotinylated analog, 2AI-3, showed expected activity against *A. baumannii* biofilms (Supporting Information). From these pull-down experiments and subsequent mass spectrometry analysis, we readily identified that the 2AI-1 biotinylated analog extracted the response regulator BfmR (Supporting Information). This interaction was confirmed using the anti-BfmR antibody for visualization. Here the pull-down experiment was repeated using (i) lysate and (ii) purified, recombinantly expressed BfmR. The antibody indicated the major protein band that bound 2AI-1 was BfmR in the lysate as well as the purified protein. In Fig. 2 a western blot illustrates a biotynilated analog of 2AI-1 used with magnetic strepavidin beads in a pull down assay, to confirm binding to the BfmR protein in *A. baumannii* lysate and recombinantly expressed BfmR.

Lane 1 shows lysate from *A. baumannii* after being exposed to the BfmR antibody. Lane 2 shows BfmR that was bound from the lysate by the 2AI-1 analog and exposed to the BfmR antibody. As a control, recombinantly expressed BfmR (Lane 5) was used in an identical pull down. Lanes 6, 7 and 8 show the recombinant protein bound the 2AI-1 analog. Subsequently fluorescence spectroscopy also corroborated that 2AI-1 bound to purified BfmR.

Like the majority of response regulators, BfmR has two domains: an N-terminal phosphporylation domain and a C-terminal DNA-binding domain. To determine which domain 2AI-1 interacts with, we performed further pull-down experiments using biotinylated 2AI-1 with BfmRN (N-terminal BfmR, residues 1–129) and BfmRC (C-terminal BfmR, residues 129–238). These experiments showed that 2AI-1 bound to both BfmRN and BfmRC (Supporting Information). This suggests that 2AI-1 compound contacts both domains when binding to the full-length protein and may reside at the inter-domain interface.

Subsequently, we performed computational docking for BfmR and 2AI-1. The high homology between response regulators allows for realistic structural models to be generated[26]. The goal was to locate a general region on BfmR where 2AI-1 might preferentially bind (i.e. with the lowest energy) and whether this location supported the pull-down data. The BfmR model was based on the high-resolution PhoP structure (PDB 3ROJ)[27]. Like BfmR, PhoP is a member of the OmpR response regulator family. It was chosen as the optimum structural template for BfmR because (i) its structure is available, (ii) its secondary structure prediction is very similar to BfmR and (iii) the linker region between the N- and C-terminal domains is of comparable length, suggesting similar tethering. Using AutoDock (MGLTools), so-called 'blind/unbiased molecular docking' was performed, using standard docking and scoring parameters, except for the degree of exhaustiveness (=100)[28]. The search space comprised the entire target PDB so that no regional biasing was evident. The docking calculations show that 2AI-1 preferentially targets the interface between the N-terminal and C-terminal domains in BfmR, with 70% of the interactions computed found to reside in the inter-domain interface. Figure 3 shows the lowest energy binding solutions for 2AI-1 binding to the BfmR model. Contacts are made between 2AI-1 and both domains.

These studies strongly support the pull-down experiments and bolster the proposition that 2AI-1 targets BfmR in such a way as to directly affect both the N-terminal regulatory domain and the C-terminal DNA-binding domain.

Finally, in order to see whether 2AI-1 has any broad preference for response regulators, we performed more pull-down experiments with the following response regulators/domains: Spo0A, Spo0AN (Spo0A N-terminal domain), Spo0AC (Spo0A C-terminal domain), ComAN (ComA N-terminal domain), ComAC (ComA C-terminal domain), Spo0F and

CheY (response regulators consisting of just a regulatory domain). In each case, binding between the 2AI-1 analog and the protein/domain was observed. Pull-down negative controls were also run. The following 'all helical' proteins were employed: VanU, LuxU, SinI and SinRN. For each, no binding was seen between the 2AI-1 analog and the protein. These studies suggest that 2AI-1 preferentially targets response regulators.

Here, we established that the response regulator BfmR from the opportunistic human pathogen *A. baumannii* is a target for the 2-aminoimidazole compound 2AI-1. BfmR controls biofilm development in *A. baumannii* and 2AI-1 inhibits biofilm formation. Pull-down experiments show that 2AI-1 binds to both the regulatory and output domains of BfmR and computational docking suggests that the putative site for interaction is at the inter-domain interface. 2AI-1 shows general targeting preference for response regulators and is able to permeate the cell membrane. As noted, there has been great interest in controlling the action of two-component systems for therapeutic advantage[29,30]. Here we suggest that a family of 2-aminoimidazole compounds target response regulators in such a way that two-component system function is hindered and the ability of bacteria to protect themselves is impeded. This allows for the possibility of using our 2AI molecules as adjuvants to existing (or new) antimicrobial treatments.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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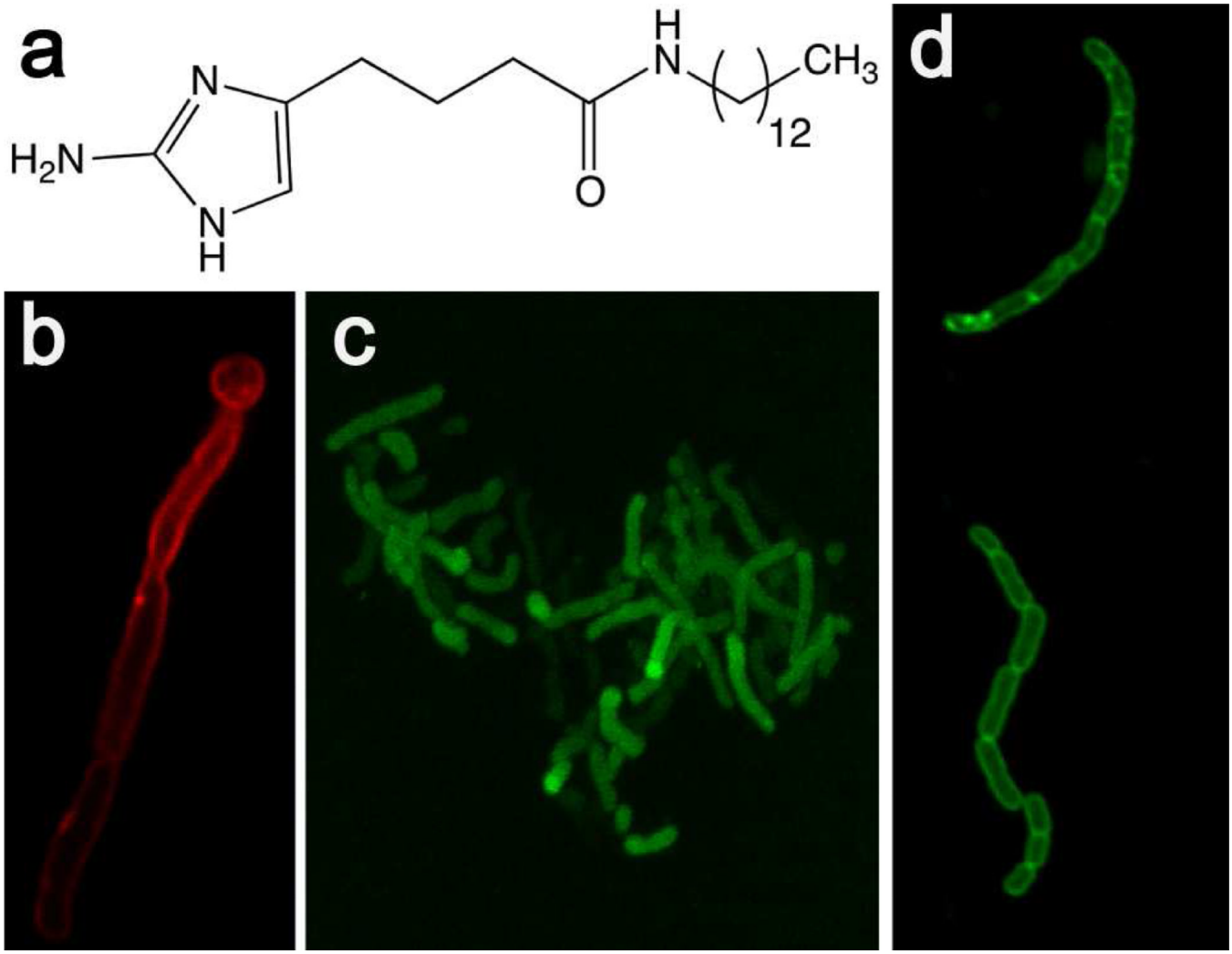

Figure 1.

(1a) Compound 2AI-1 used in these studies ; (1b) *A. baumannii* cells exposed to octadecyl-rhodamine B; (1c) live *A. baumannii* treated with the 2AI-1 fluorescein analog; (1d) formaldehyde-fixed *A. baumannii* treated with the 2AI-1 fluorescein analog.

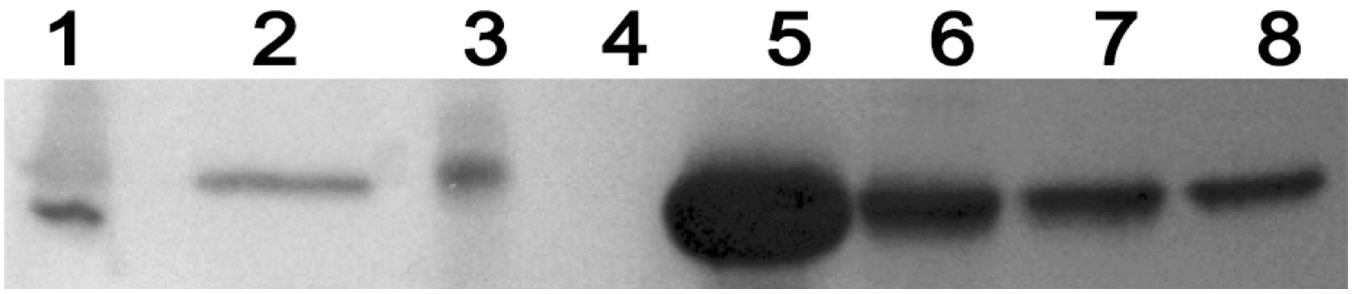

Figure 2.
Western blot confirming biotinylated 2AI-1 binding to BfmR in *A. baumannii* lysate (lane 2 – bound by 2AI-1) and BfmR recombinantly expressed (lanes 6–8). Lane 1 – lysate (exposed to anti-body); lane 3 - molecular weight marker; lane 4 - space; lane 5 BfmR standard.

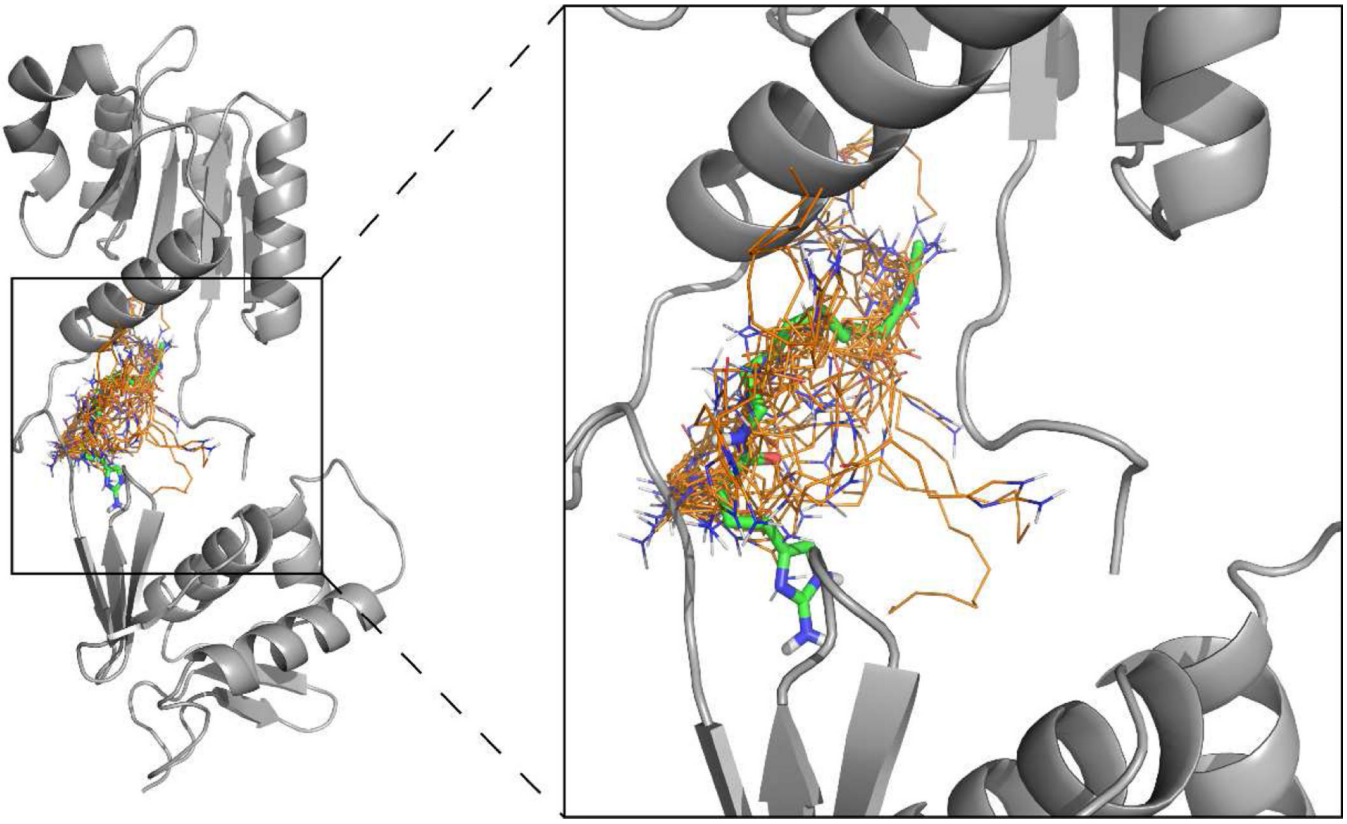

Figure 3.
Highest populated cluster of structures for the 2AI-1:BfmR-modeled interaction. In gray is the BfmR modeled structure; in orange is the highest populated cluster of 2AI-1 structures. Shown in green is the lowest energy docked structure for this cluster. Of the docked solutions interacting with BfmR between the N- and C-termini, this cluster represents ~70% of these docked solutions.