Small Molecule Inhibitors of the BfrB–Bfd Interaction Decrease *Pseudomonas aeruginosa* Fitness and Potentiate Fluoroquinolone Activity

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**ABSTRACT:** The iron storage protein bacterioferritin (BfrB) is central to bacterial iron homeostasis. The mobilization of iron from BfrB, which requires binding by a cognate ferredoxin (Bfd), is essential to the regulation of cytosolic iron levels in *P. aeruginosa*. This paper describes the structure-guided development of small molecule inhibitors of the BfrB–Bfd protein–protein interaction. The process was initiated by screening a fragment library and followed by obtaining the structure of a fragment hit bound to BfrB. The structural insights were used to develop a series of 4-(benzylamino)- and 4-((3-phenylpropyl)amino)-isoindoline-1,3-dione analogs that selectively bind BfrB at the Bfd binding site. Challenging *P. aeruginosa* cells with the 4-substituted isoindoline analogs revealed a dose-dependent growth phenotype. Further investigation determined that the analogs elicit a pyoverdin hyperproduction phenotype that is consistent with blockade of the BfrB–Bfd interaction and ensuing irreversible accumulation of iron in BfrB, with concomitant depletion of iron in the cytosol. The irreversible accumulation of iron in BfrB prompted by the 4-substituted isoindoline analogs was confirmed by visualization of BfrB-iron in *P. aeruginosa* cell lysates separated on native PAGE gels and stained for iron with Ferene S. Challenging *P. aeruginosa* cultures with a combination of commercial fluoroquinolone and our isoindoline analogs results in significantly lower cell survival relative to treatment with either antibiotic or analog alone. Collectively, these findings furnish proof of concept for the usefulness of small molecule probes designed to dysregulate bacterial iron homeostasis by targeting a protein–protein interaction pivotal for iron storage in the bacterial cell.

**INTRODUCTION**

Antibiotic resistant infections are a worldwide threat to public health. The challenge posed by the emergence of antibiotic resistant strains is compounded by slow to nearly stalled development of new antibiotics and validation of new targets.1−3 Hence, antibiotic resistant infections have the potential to undermine many achievements in modern medicine, such as organ transplantation, major surgery, and cancer chemotherapy. The World Health Organization (WHO) published a priority list for research and development of new antibiotics to combat multidrug resistant bacteria, and assigned critical priority to the Gram-negative carbapenem-resistant *Acinetobacter baumanii* and *Pseudomonas aeruginosa*, and third-generation cephalosporin resistant Enterobacteriaceae.4 *P. aeruginosa* is one of the leading Gram-negative pathogens associated with hospital infections due to their propensity to colonize urinary catheters and endotracheal tubes5,6 and accelerate lung function decay that lowers the survival of cystic fibrosis patients.7,8 Responding to this call requires vibrant research and continued investment in the early stages of drug development, in order to ensure a pipeline of novel ideas and approaches.9 In this context, strategies that interfere with bacterial iron acquisition and homeostasis are...
regarded as having potential as new therapeutic interventions.9−13 Iron is essential for bacteria because of its involvement in multiple metabolic processes, including respiration and fundamental enzymatic reactions.14 Pathogenic bacteria must obtain iron from the host, but host nutritional immunity maintains extremely low concentrations of free iron, thus denying the essential nutrient to invading pathogens.15−18 In addition, the very low solubility of the ferric ion (Fe3+) severely limits its bioavailability, and the reactivity of the soluble ferrous iron (Fe2+) toward hydrogen peroxide and oxygen induces oxidative stress. Consequently, the processes of bacterial iron homeostasis (acquisition, storage and utilization) are highly regulated to ensure sufficiency for metabolic needs while preventing iron-induced toxicity.19,20 Herein, we describe a new approach to dysregulate iron homeostasis in P. aeruginosa that utilizes small molecule probes designed to block the interaction between the iron storage protein bacterioferritin B (BfrB) and its cognate partner, the bacterioferritin-associated ferredoxin (Bfd).

Bacteria store iron reserves in bacterial ferritin (Ftn) and in bacterioferritin (Bfr).21−23 The roughly spherical and hollow structures of Bfr and bacterial Ftn, which are formed from 24 identical subunits, have an outer diameter of ~120 Å, an inner diameter of ~80 Å, and an interior cavity that can store up to ~3000 iron ions in the form of a Fe3+ mineral (Figure 1A). Bfrs, which exist only in bacteria, bind 12 heme groups buried in the 12 Bfd binding sites are equivalent and independent, and that Bfd binds to BfrB with a Kd of approximately 3 μM.31 These investigations also revealed that M1, Y2, and L5 in Bfd form a continuous set of interactions with L68 and E81 in BfrB, which contribute significantly to the stabilization of the BfrB−Bfd complex (Figure 1C). In agreement, the Kd values for the association between Bfd and the L68A or E81A mutants of BfrB are approximately 100-fold larger, and the association between Bfd and the BfrB L68A/E81A double mutant is undetectable.31

The repercussions of blocking the BfrB−Bfd interaction on P. aeruginosa iron metabolism have been investigated by deleting the bfd gene. These investigations, which showed an irreversible accumulation of Fe3+ in BfrB with concomitant iron deprivation in the cytosol, established the BfrB−Bfd interaction as a novel target to rationally induce iron homeostasis dysregulation in bacteria.19 Consequently, it is important to discover small molecule inhibitors of the BfrB−Bfd interaction, which can be used as chemical probes to study bacterial iron homeostasis and uncover additional vulnerabilities in the bacterial cell exposed by iron metabolism dysregulation. Chemical probes are a powerful complement to the utilization of genetic techniques because they offer dose-dependent, selective, and temporal control over target proteins, which can be utilized in combination with other synergistic or antagonistic probes.32,33 Herein we present the results from a structure-guided program aimed at the development of small molecules designed to inhibit the BfrB−Bfd interaction in P. aeruginosa. These novel probes are capable of penetrating the bacterial cells, where they inhibit the mobilization of iron from BfrB and elicit perturbations in iron homeostasis that decrease bacterial fitness, and also potentiate the bactericidal activity of fluoroquinolone antibiotics.

### EXPERIMENTAL SECTION

#### Chemicals, Strains, and Growth Media

Chemicals were purchased from Fisher Scientific unless otherwise stated. *Pseudomonas aeruginosa* (PAO1) was purchased from the University of Washington Genome Center. The PAO1-derived strain with an unmarked, in-frame deletion of the *bfrB* gene had been prepared previously.33 *P. aeruginosa* clinical isolates (MR3B and MR60) were obtained from Seattle Children’s Research Foundation. The clinical isolates of *P. aeruginosa* (strains isolated from the lungs of people with cystic fibrosis) were obtained from Seattle Children’s Research Institute via the Antimicrobial Tools and Resources (Microbial Resources)
services offered by the Cystic Fibrosis Foundation ([https://www.cff.org/Research/Research-Resources/Tools-and-Resources/Antimicrobial-Tools-and-Resources/]). All strains were kept on Pseudomonas Isolation Agar (PIA) (BD Biosciences, CA). M63 media was prepared as previously reported, with a small modification. It contained per liter the following: 2 g of (NH₄)₂SO₄, 13.6 g of KH₂PO₄, 2 g of glucose, 4 g of citric acid, 0.25 g of tryptophan (Acros organics), 5 g of nontechmical grade casamino acids (BD scientific), and 0.24 g of MgSO₄ (Alfa Aesar), and the pH was adjusted to 7.0 with KOH. The M63 media also contained 0.1% (w/v) of hypermellose (HPMC, Sigma-Aldrich) to prevent aggregation of the analogs in aqueous solution. Colorimetric analysis showed that the M63 media contains 2 μM Fe. When necessary, the M63 media was supplemented with additional iron by addition of a small volume of 1 mM (NH₄)₂Fe(SO₄)₂ (pH ≈ 2.0) to give the desired final iron concentration.

Fragment Library Screening Using Saturation Transfer Difference (STD) NMR Spectroscopy. Experimental details are presented in the Supporting Information.

Synthesis and Preparation of Analogs. Experimental details of the synthetic procedures developed to prepare compounds to be tested as inhibitors of the BfrB–Bfd interaction, as well as the corresponding 1H and 13C NMR spectra, are presented in the Supporting Information.

Crystallization, Ligand Soaking, and Data Collection. Crystallization screening was conducted in Compact 300 (Rigaku Reagents) sitting drop vapor diffusion plates at 18 °C using equal volumes (0.5 μL) of BfrB and crystallization solution equilibrated against 75 μL of the latter. Three different BfrB constructs were investigated to grow crystals of BfrB suitable for soaking experiments with the different fragments and analogs. BfrB crystals were observed in 1–2 days as follows: C89S/K96C BfrB: Red prismatic crystals were obtained from Wizard 1–2 (Rigaku Reagents) condition E2 (35% (v/v) 2-methyl-2,4-pentanediol, 100 mM MES 6.5, 200 mM Li₂SO₄). Ago-BfrB (BfrB devoid of heme): Colorless prismatic, or light yellow, crystals were obtained from the Wizard 3–4 screen (Rigaku Reagents) condition B1 (8% (v/v) PEG 8000, 100 mM Na acetate pH 4.6). BfrB: Red plates grew from the Cryo 1–2 HT screen (Rigaku Reagents) condition H6 (30% (v/v) PEG 200, 100 mM Na acetate pH 4.5, 100 mM NaCl). To prepare for soaking experiments, a stock solution (100 mM in DMSO) of each fragment or analog (compound) was mixed with crystallization solution to obtain a 20 mM compound solution to be used in soaking experiments. Crystals were transferred to these soaking solutions and incubated for 3.0 to 3.5 h before harvesting directly from the drop and storing in liquid nitrogen. Analog 13 was soaked in a 25 mM compound solution for 2 h, and analog 16 was soaked in a 10 mM compound solution for 3 h. The compound soaking solutions, which also served as the cryoprotectant, contained 80% crystallization solution and 20% DMSO. Structures of compounds bound to BfrB were obtained from the following compound/BfrB crystal combinations: Fragment 1/C89S/K96C BfrB, analog 12/ago-BfrB, and analogs 11, 13, 14, 15, 16/BfrB. X-ray diffraction data were collected at the Advanced Photon Source beamline 17-ID using a Dectris Pilatus 6 M pixel area detector.

Structure Solution and Refinement. Intensities were integrated using XDS, via Autocrop, and the Laue class analysis and data scaling were performed with Aimless. Structure solution was conducted by molecular replacement with Phaser, and structure refinement and manual model building were performed with Phenix and Coot, respectively. Electron density omit maps for the ligands were calculated using the Polder omit routine with the Phenix software suite. Structure validation was carried out with Molprobity, and figures were prepared with CCP4mg. The search models used for molecular replacement were as follows: C89S/K96C BfrB (PDB: 4FDQ) and BfrB (PDB: SDQ). Ago-BfrB: Structure solution was carried out using a single subunit of a previously determined structure of BfrB as the search model (PDB: 3IS7). The top solution was obtained in the space group C222, with 12 molecules in the asymmetric unit.

Analysis of Secreted Pyoverdin. These experiments were carried out in 96 well plates, as described above. P. aeruginosa cells treated with analog 11 or 16 (125 μM final concentration) were cultured for 13 h before the contents of each well were serially diluted in phosphate buffered saline (PBS, pH 7.4) and then plated on PIA plates to enumerate viable cells. The 50-fold diluted solution was centrifuged, and the cell-free supernatant was analyzed for pyoverdin by fluorescence spectrophotometry in a Synergy H1 microplate reader (Biotek) with excitation at 400 nm and emission at A₅₅₅ = 455 nm. Full emission spectra (430–550 nm) were also recorded using a PerkinElmer LS50B spectrophotometer.

Imaging of Iron Stored in BfrB and Analysis of Total Iron Levels. Precultures were grown as described above, and cells were diluted in M63 media supplemented with 4 μM Fe (6 μM total Fe) to O₅₀ = 0.1 in 50 mL conical tubes. The resultant cell suspensions (50 mL) were mixed with a small volume of a 10 mM working solution of analog 16 in DMSO to give a 125 μM solution and 2% DMSO, or simply 2% DMSO (control). The conical tubes were covered with an air permeable membrane, and the cultures were incubated at 35 °C and 120 rpm for 6, 9, 12, 15, 18, 21, and 24 h. Prior to separating the cells by centrifugation (4000 rpm, 15 min), a 100 μL aliquot was withdrawn from each conical tube and serially diluted for plating and enumerating viable cells. The separated pellet was washed with 5 mL of PBS and frozen at −20 °C for subsequent analysis. The cell pellets were used to image iron stored in BfrB according to a previously described method with some minor modifications. Briefly, cell pellets were suspended in 300 μL of lysis buffer (50 mM Tris pH 8.0, 20% glycerol, 2 g/mL lysozyme, 0.2 mg/mL DNase, 100 mM NaCl, 10 mM MgCl₂, and 1% Triton X) and freeze–thawed twice.
using liquid N₂. The resultant suspensions were incubated at 25 °C for 90 min in a rocker and then centrifuged at 12 500 rpm for 15 min at 4 °C. Lysate solutions (100 μL) were each mixed with 10 μL of the loading dye, and the samples (100 μL) were loaded onto a 3-mm-thick native polyacrylamide gel (8% resolving gel and 4% stacking gel) for separation. Electrophoretic separation was carried out at 60 V for 7 h at 4 °C, and the gels were stained with Ferene S \( \text{S}^2^- \) for 10 min in a solution containing 0.049 g of Ferene S, 250 μL of thioglycolic acid, 2.5 mL of acetic acid, and 100 mL of water. The scanned images were processed and compared using ImageJ.48

To determine the total iron levels of cells treated with analog 16 or DMSO only (control), 5 mL cultures were grown in M63 media supplemented with 4 μM iron, as described above, and analyzed at 12, 15, 21, 24, and 28 h. Prior to separation of the cells by centrifugation (4000 rpm, 15 min), a 100 μL aliquot was withdrawn from each conical tube and serially diluted for plating, in order to enumerate viable cells. Cell pellets were washed twice with 10 mL of PBS, and the total levels of cellular iron were measured using a published protocol.19,49 In brief, cell pellets were mixed with 500 μL of freshly prepared digestion reagent (1:1 v/v 1.2 N HCl and 4.5% w/v KMnO₄ in water), thoroughly mixed by vortexing, and incubated at 65 °C for 4 h. The digested solutions were cooled to 25 °C, mixed with 500 μL of iron chelating agent (6.5 mM Ferene S, 13.1 mM neocuproine, 2 M ascorbic acid, 5 M ammonium acetate), and then incubated at 25 °C for 30 min. The iron concentration of the resultant solution was measured from the absorbance of the Fe²⁺–Ferene S complex at 593 nm (ε = 34,500 M⁻¹ cm⁻¹)50 using a Cary 60 UV−vis spectrophotometer, normalized by cell count and reported as Fe atoms per CFU (colony forming unit). The colorimetric determination of total intracellular iron offers a sensitive, accurate, and low cost analytical technique, which has been shown to produce results similar to those obtained by atomic inductively coupled plasma-mass spectrometry.48

Effect of Analogs on the Potency of Fluoroquinolone Antibiotics. Cultures were treated with (i) analog 16 only; (ii) ciprofloxacin (0.25 μg/mL), levofloxacin (0.5 μg/mL), or norfloxacin (0.9 μg/mL) only; and (iii) both fluoroquinolone (concentration as above) and analog 16. Precultures were grown as described above; the cells were then diluted in M63 media supplemented with 4 μM Fe to OD₆₀₀ = 0.1 in 50 mL conical tubes. The resultant cell suspensions (5 mL) were mixed with a small volume of a 10 mM working solution of PIA for enumeration of viable cells. To treat cells with ciprofloxacin only, the procedure was identical except that the cell suspensions (5 mL) were mixed with a small volume of ciprofloxacin working solution (100 μg/mL) to give the desired antibiotic concentration and 2% DMSO. The combined treatment was carried out similarly, ensuring that the final concentration of DMSO was 2%. Measurement of Dissociation Constants (Kₐ). Experimental details are presented in the Supporting Information.

RESULTS

Screening and Detection of Fragment Binding to the Bfd Binding Site on BfrB. Structural information obtained from the BfrB–Bfd complex was used to design a fragment library to screen for molecules that bind BfrB at the Bfd-binding site. Bfd residues M1, Y2, and L5 and BfrB residues L68 and E81 dominate the buried surface area at the protein–protein interface (Figure 1C) and contribute significantly to the binding energy of the BfrB–Bfd complex.31 Consequently, the fragment library was focused on fragments that may bind at the sites occupied by Y2 and L5 from Bfd and included groups with chemical properties similar to the aromatic and aliphatic side chains of Tyr and Leu, also utilizing standard fragment criteria (MW < 300 Da, clogP < 3, and total count of hydrogen bond acceptors/donors < 3). In addition, fragments capable of π−π stacking were included with both electron-rich and -deficient aromatic rings. To screen the library in search of fragments that bind BfrB at the Bfd-binding site we developed a competition assay that utilizes saturation transfer difference (STD) NMR spectroscopy. This technique is ideally suited to screen fragments that bind to the large BfrB (∼440 kDa) because protein resonance assignments are not necessary and very low protein concentrations are required. In addition, the large rotational correlation time (τᵣ) of BfrB enhances spin diffusion and therefore saturation transfer within the protein and to the ligand.52 Two solutions were prepared for each fragment: a solution of the fragment alone, and a solution of the fragment and BfrB. Three spectra were obtained for each fragment, as illustrated in Figure S1 of the Supporting Information for fragment 1, which binds BfrB at the Bfd-binding site. The ¹H spectrum of the fragment alone was used to determine fragment integrity and solubility, the ¹H spectrum of the fragment in the presence of BfrB was used to corroborate that fragment integrity and solubility is not affected by BfrB, and the STD spectrum of the solution containing the fragment and BfrB was used to assess fragment binding. This strategy uncovered 18 compounds that bind BfrB. The specificity criterion for fragment binders, however, is that these bind BfrB specifically at the Bfd-binding site. To eliminate nonspecific binders, a displacement strategy was implemented that utilizes Bfd as a specific competitor. For this purpose, an STD spectrum was acquired from a solution containing fragment, BfrB and Bfd. Nearly complete disappearance of the STD signal indicates that the fragment binds BfrB at the Bfd-binding site. With the aid of this competitive displacement strategy, it was determined that of the 18 fragments that bind BfrB, the 6 molecules shown in Chart 1 bind at the Bfd-binding site. These fragments were advanced to the next stage, which was focused on uncovering structural information on fragment binding. Results from these experiments are presented below.

Structure-Based Optimization of Fragment Binders. A structure-guided approach was used to synthetically elaborate fragments discovered to bind BfrB at the Bfd-binding site.
site into analogs capable of binding with higher affinity. The affinity and selectivity of the fragments and analogs for the Bfd-binding site on BfrB were investigated by X-ray crystallography, surface plasmon resonance (SPR), and fluorescence polarization methods. The structures of fragments and derived analogs which have been demonstrated to bind BfrB at the Bfd-binding site using X-ray crystallography are shown in Figure 3B. During the course of these experiments, crystals obtained from three different BfrB constructs were tested in ligand soaking experiments, in an effort to identify the “best” crystals for the study. Hence, crystals of BfrB (PDB 5D8O), C98S/K96C BfrB (PDB 4TOF), and apo-BfrB were soaked in crystallization solution containing the various analogs. The structure of 5-hydroxyaminoisoindoline-1,3-dione (fragment 1) bound to BfrB was obtained by soaking crystals of C98S/K96C BfrB in a solution of the fragment, as described in the Figure 3B. Protein–protein interaction interface of the BfrB–Bfd complex. Subunits A and B of a BfrB subunit dimer are colored gray and green, respectively. (A) Residues Y2 and L5 of Bfd (cyan cylinders) are positioned within pockets at the BfrB subunit dimer interface. (B) Fc – F, omit map (orange mesh) contoured at 3σ showing 5-hydroxyaminoisoindoline-1,3-dione (fragment 1) bound within the cleft occupied by L5 in the BfrB–Bfd complex. (C) Hydrogen bond interactions (dashed lines) between fragment 1 and BfrB. Water mediated contacts are indicated by the solid lines.

Figure 3. (A) Schematic summary of the synthetic procedures employed to prepare analogs of fragments 1, 5, and 8 to form ethers 4 derived from 5-hydroxyisoindoline-1,3-dione (1); amines 6 or amides 7 derived from 5-aminoisoindoline-1,3-dione (5); or amines 9 or amides 10 derived from 4-aminoisoindoline-1,3-dione (8). Descriptions of the synthetic procedures, as well as the characterization of the analogs are presented in the Supporting Information. (B) Structures of fragment 1 and analogs prepared from fragment 8, which have been shown to bind BfrB at the Bfd binding site by X-ray crystallography.

Figure 2. Protein–protein interaction interface of the BfrB–Bfd complex. Subunits A and B of a BfrB subunit dimer are colored gray and green, respectively. (A) Residues Y2 and L5 of Bfd (cyan cylinders) are positioned within pockets at the BfrB subunit dimer interface. (B) Fc – F, omit map (orange mesh) contoured at 3σ showing 5-hydroxyaminoisoindoline-1,3-dione (fragment 1) bound within the cleft occupied by L5 in the BfrB–Bfd complex. (C) Hydrogen bond interactions (dashed lines) between fragment 1 and BfrB. Water mediated contacts are indicated by the solid lines.
Experimental Section. In subsequent experiments, crystals of apo-BfrB were used because this protein formed robust, highly reproducible crystals. These experiments culminated in the structure of analog 12 bound to BfrB. It is important to underscore that the structure of apo-BfrB is nearly identical to that of BfrB, at the subunit level (RMSD = 0.18 Å), as well as the biological assembly level, including the Bfd-binding sites (Figure S2A). Additional evidence indicating that the Bfd-binding sites on apo-BfrB are unaffected relative to the holo-protein was obtained in the dissociation constant for the interaction between Bfd and apo-BfrB ($K_d = 3.1 \mu M$) (Figure S2B and C), which is nearly identical to that previously reported for the interaction between Bfd and holo-BfrB ($K_d = 3.4 \mu M$). Finally, the BfrB protein also produced reproducible crystals that were isomorphs with those formed by apo-BfrB. Consequently, the structures of analogs 11 and 13–16 bound to BfrB were obtained by soaking crystals of BfrB in solutions of each of the analogs.

Figure 2A depicts a portion of the BfrB–Bfd complex interface and illustrates how Y2 and L5 in Bfd (cyan cylinders) contribute to anchor Bfd to the BfrB surface. A prior study indicated that interactions between L68 and E81 in BfrB and Y2 and L5 in Bfd contribute significantly to the binding energy of the BfrB–Bfd complex. It was therefore surmised that fragments capable of binding to this region of the BfrB surface would be good candidates for subsequent structure-guided synthetic elaboration aimed at discovering inhibitors of the BfrB–Bfd interaction. To obtain the structures of fragments bound to BfrB, crystals of the protein were soaked in crystallization solution containing each of the 6 fragments found to bind BfrB at the Bfd-binding site with the aid of the competitive displacement STD NMR strategy described above. These efforts culminated in a 1.5 Å resolution cocystal structure of 5-hydroxyisoindoline-1,3-dione (1) bound to BfrB (Figure 2B and 2C and Table S1), which showed that 1 binds BfrB at the Bfd-binding site, in the same pocket where L5 from Bfd would bind. The fragment rests on a platform at the base of a shallow depression on the BfrB surface formed by the side chains of L68 and F70B (the subscript denotes subunit, and the superscript denotes residue number), surrounded by a semicircular wall comprised by the side chain and backbone atoms of L68A, N70A, Q72A, and L74A. One of the carbonyl-oxygen atoms of 1 accepts a H-bond from the backbone NH of L74A, and its N–H group forms a H-bond to the backbone carbonyl of P74A. Additional stabilization for binding probably stems from the network of H-bonded waters linking a carbonyl oxygen in 1 and G81A in BfrB. This structural information suggested a strategy to grow 1, or its analogs 4-aminoisoindolone-1,3-dione (8) and 5-aminoisoindolone-1,3-dione (5), by branching from the isoindoline ring carbons C4 or C5 to engage the cleft formed by the side chains of L68 and E81 in BfrB, where Y2 from Bfd anchors.

A general synthetic approach (Figure 3A) was formulated to generate a series of ether analogs represented by 4, amine analogs represented by 6 or 9, and amide analogs represented by 7 or 10. Preparation of the ether analogs 4 started with the esterification of the acid groups in 4-hydroxyphthalic acid 2, followed by alkylation of the phenolic oxygen to produce 3 and subsequent base cleavage of the esters and cyclization to produce the isoindoline-1,3-dione ethers 4. Synthesis of the amine analogs represented by 6 and 9 was carried out by reductive amination of 5 or 8, respectively, with a series of aldehydes, whereas amide analogs 7 and 10 were obtained from 5 or 8, respectively, via reactions with a series of acid chlorides. The collection of ether, amine, and amide analogs prepared for this study, as well as the details of their synthetic preparation and characterization, are presented in the Supporting Information.

Although all of the compounds synthesized for each of the analog types shown in Figure 3A were tested in crystal soaking experiments, structures of analog-bound BfrB were obtained only for analogs 11–16 (Figure 3B), which are derivatives of 4-aminoisoindoline-1,3-dione (8) with $-(\text{CH}_2)_n-$ and $-(\text{CH}_2)_o-$ linkers. The pose of each of these analogs in the Bfd-binding site of BfrB is shown in Figures 4 and S3.

Figure 4. Binding modes of analogs 11 and 16 at the Bfd-binding site on BfrB. Subunits A and B of a BfrB subunit dimer are colored gray and green, respectively. (A) $F_A - F_B$ omit map (orange mesh) contoured at 3σ from analog 11 bound at the Bfd-binding site on BfrB. (B) Same as panel A but showing a different perspective to illustrate the two orientations modeled for the o-hydroxyphenyl ring of analog 11. (C) Hydrogen bond interactions (dashed lines) between analog 11 and BfrB. Water mediated contacts are indicated by the solid lines. (D) $F_A - F_B$ omit map (orange mesh) contoured at 3σ from analog 16 bound at the Bfd-binding site on BfrB. (E) Same as panel D but rotated to illustrate the m-hydroxyphenyl ring of analog 16 positioned between the cleft formed by the side chains of L68A and E81B in BfrB. (F) Hydrogen bond interactions (dashed lines) between analog 16 and BfrB. Water mediated contacts are indicated by the solid lines.

Inspection reveals that the isoindoline-1,3-dione moiety in all the compounds invariably binds BfrB in a manner identical to that described above for fragment 1 binding to BfrB (see Figure 2). In addition, and as was expected, the benzyl portion of the analogs extends to engage the cleft formed by the side chains of L68A and E81B in BfrB via hydrophobic packing interactions. The interactions experienced by analog 11 at the Bfd-binding site on BfrB are illustrated in Figure 4A–C. Strong electron density consistent with the compound can be observed in 9 of the 12 subunits in the asymmetric unit; the 3 subunits in which compound 11 was not modeled displayed electron density not associated with the protein at the Bfd-binding site, but this electron density was too weak to model the ligand. The o-hydroxyphenyl ring of 11 is observed in two orientations that differ by a 180° rotation of the ring; one
orientation places the hydroxyl group pointing toward the base of the cleft, where it forms a hydrogen bond with $G_B^{80}$, while the second orientation places the hydroxyl group toward the solvent and enables packing of the $o$-hydroxyphenyl ring and the $-(CH_2)_2-$ moiety bridging the phenyl and isonindoline rings against the side chains of $M_B^{31}$ and $I_B^{79}$, respectively. Additional stabilization for binding probably stems from the network of H-bonded waters linking a carbonyl oxygen in $11$ and $C_B^{67}$, similar to the network of H-bonded water molecules observed in the structure of $1$ bound to BfrB (see Figure 2C). The structure of a similar analog ($12$) bound to BfrB shows identical interactions of the isonindoline-1,3-dione moiety with BfrB and similar interactions of the phenyl ring with the cleft formed by the side chains of $L_A^{68}$ and $E_B^{81}$ on the BfrB surface (Figure S3B–C).

A similar close view of compound $16$ bound at the Bfd binding site on BfrB (Figure 4D–F) illustrates how the isonindoline-1,3-dione ring presents the same pose and set of interactions as those described above for fragment $1$ and analogs $11$ and $12$. Clear electron density consistent with the compound can be observed in all $12$ subunits in the asymmetric unit, but the $m$-hydroxyphenyl ring was partially disordered in several of the subunits. The $m$-hydroxyphenyl ring and the $-(CH_2)_2-$ linker pack against the $L_A^{68}$ and hydrophobic portion of the $E_B^{81}$ side chains, with additional stabilization probably stemming from a network of H-bonded waters connecting a carbonyl oxygen in the isonindoline-1,3-dione ring, the hydroxyl group on aromatic ring, and the carbonyl oxygen in $L_A^{68}$. The structures of four additional compounds similar to $16$ bound to BfrB (Figure S3), which show a nearly identical pose of the isonindoline-1,3-dione moieties and very similar interactions of the linkers and phenyl rings, demonstrate the specificity with which the series of 4-amino derivatives listed in Table 1 engage the Bfd-binding site on BfrB.

Prior to testing the effect that the 4-substituted isonindoline-1,3-dione derivatives might exert on $P. aeruginosa$ cells, the strength of their interaction with BfrB was evaluated in vitro with a fluorescence polarization assay developed based on the intrinsic fluorescence of the isonindoline-1,3-dione moiety. Because initial fluorescence spectroscopic measurements revealed that the heme groups in BfrB interfere with the signal of the fluorescent ligand, we utilized apo-BfrB for these measurements, capitalizing on our earlier findings that the Bfd-binding sites in apo-BfrB are nearly identical to those in BfrB, and that the $K_d$ for the interaction between apo-BfrB and Bfd is very similar to that measured for the interaction between BfrB and Bfd (see above and Figure S2). Hence, the $K_d$ values were measured by titrating apo-BfrB into a fixed concentration of the appropriate fluorescent 4-aminoisondoline-1,3-dione ligand while analyzing fluorescence polarization and intensity near the emission $\lambda_{max}$ (Figure S4). The $K_d$ values (Table 1) show that

| Analog | Structure | $K_d$ (µM) | $IC_{50}$ (µM) |
|--------|-----------|------------|---------------|
| 8      | ![Structure](image8) | 300 ± 50   | not active    |
| 11     | ![Structure](image11) | 11 ± 1     | 258 ± 23      |
| 12     | ![Structure](image12) | 15 ± 2     | not active    |
| 13     | ![Structure](image13) | 3 ± 1      | 201 ± 18      |
| 14     | ![Structure](image14) | 4 ± 2      | 143 ± 8       |
| 15     | ![Structure](image15) | 5 ± 2      | 227 ± 10      |
| 16     | ![Structure](image16) | 6 ± 1      | 121 ± 4       |

Table 1. Structure, Binding Affinity, and $IC_{50}$ of 4-Aminoisonindoline-1,3-dione Derivatives
analogs 11–16 exhibit significantly higher affinity than fragment 8, observations that are in agreement with the structural information, which revealed that growing the fragments from the isoindoline ring carbon C4 make it possible to engage the cleft formed by the side chains of L68 and E81 in BfrB. The two derivatives with a \(-(\text{CH}_2)_3\) linker exhibit binding affinities approximately 2- to 5-fold lower than derivatives with a \(-(\text{CH}_2)_2\) linker, also in agreement with a relatively more efficient hydrophobic packing facilitated by the longer linker.

### 4-Aminoisoindoline-1,3-dione derivatives elicit a growth retardation phenotype in *P. aeruginosa* cells.

The binding affinity and structural information indicate that most of the analogs in Table 1 bind BfrB at the Bfd binding site with a strength comparable to that of the BfrB–Bfd association (\(K_d = 3 \mu M\)), therefore suggesting that these compounds may be capable of interfering with the BfrB–Bfd interaction in the *P. aeruginosa* cytosol. As will be described below, this idea was investigated, first by demonstrating that the analogs elicit a growth phenotype in *P. aeruginosa*, and then by showing that one of the most potent analogs inhibits the mobilization of iron from BfrB in the bacterial cytosol.

To investigate the effect of the analogs on cell growth, cultures of *P. aeruginosa* in M63 media were challenged with 4-aminoisoindoline-1,3-dione derivatives, and their growth was monitored in 96 well plates by following the OD\(_{600}\) (Figure 5A and 5B). As expected, the analogs elicit a monophasic dose-dependent growth defect on the wild type cells. In comparison, the analogs induce a biphasic growth response on the ΔbfrB mutant cells, which consists of a shallow first phase (0–100 \(\mu M\)) that is nearly independent of analog concentration, followed by a steep second phase where the analogs become rapidly toxic. Since BfrB is not essential, it is likely that the cell...
can compensate for the absence of the iron storage protein. Nevertheless, the nearly independent growth defect observed in the first phase, which is in good agreement with the absence of BfrB, supports the idea that analogs 11 and 16 exhibit significant selectivity for BfrB in the P. aeruginosa cell. The sudden onset of toxicity observed in the second phase, which is not observed with the wild type cells, is probably related to off-target effects that expose a fitness vulnerability caused by the absence of BfrB. Consequently, it is possible to conclude that the dose-dependent growth defect elicited by 11 or 16 on wild type P. aeruginosa cells is largely a consequence of the interaction between the small molecule inhibitors and BfrB in the bacterial cell. This issue was investigated in additional detail by probing the phenotypic and biochemical response of wild type cells treated with the small molecule inhibitors. The results of these studies are presented below.

4-Aminoisoindoline-1,3-dione derivatives engage their target (BfrB) in P. aeruginosa cells. Studies...
ciprofloxacin. (A) P. aeruginosa PA01 cultures in M63 media supplemented with 4 μM iron were treated with analog 16 only, fluoroquinolone at the reported MIC only, and a combination of analog 16 and fluoroquinolone at the MIC. The fluoroquinolones studied are (A) ciprofloxacin (0.25 μg/mL), (B) levofloxacin (0.5 μg/mL), and (C) norfloxacin (0.9 μg/mL). (D) P. aeruginosa clinical isolate MR3B cultures in M63 media supplemented with 4 μM iron were treated with analog 16 only, or ciprofloxacin only (0.2 μg/mL), or a combination of 16 and ciprofloxacin. (E) P. aeruginosa clinical isolate MR60 cultures in the same media as above were treated with analog 16, or ciprofloxacin (1.0 μg/mL), or a combination of 16 and ciprofloxacin. Error bars represent the standard deviation from three independent experiments.

conducted to determine whether the analogs are capable of engaging BfrB and inhibiting iron mobilization from the bacterioferritin in the P. aeruginosa cytosol were conducted mainly with the most efficacious analog (16), and when practical, also with analog 11, which is the most active of the two compounds harboring a −(CH₂)− linker. As indicated above, genetic manipulations were used in prior work to delete the bfd gene (Δbfd) and evaluate the consequences of inhibiting the BfrB−Bfd interaction in P. aeruginosa. Preventing the BfrB−Bfd interaction in the Δbfd mutant cells dysregulates iron homeostasis by causing the irreversible accumulation of iron in BfrB and the concomitant depletion of free iron levels in the cytosol. The resultant phenotype is overproduction of the siderophore pyoverdin, which is ~4-fold larger than that secreted by wild type cells. If the 4-aminoisoindoline-1,3-dione analogs are capable of binding BfrB, blocking the BfrB−Bfd interaction, and consequently inhibiting iron mobilization from BfrB in the P. aeruginosa cytosol, then they would be expected to elicit a similar pyoverdin hyper-production phenotype. To investigate whether 4-aminoisoindoline-1,3-dione derivatives indeed elicit such a phenotype, P. aeruginosa cells cultured in M63 media were treated with analog 16 at a concentration of 125 μM. As expected, cell cultures treated with the analog exhibited ca. 30% of viable cells relative to cells in the untreated control (Figure 7A). To analyze the levels of secreted pyoverdin, cells were pelleted and the supernatant was diluted 500-fold prior to measuring the fluorescence intensity at 455 nm (Figure 7B). Normalizing the intensity of pyoverdin fluorescence to cell density (CFU/mL) shows that cells treated with 16 secrete ~4-fold more pyoverdin than the untreated control (Figure 7C). To demonstrate that the intrinsic fluorescence of 16, which is significantly weaker than that of pyoverdin, does not interfere with the measurement, the fluorescence spectrum from a solution of analog 16 (125 μM) after a 500-fold dilution in PBS is shown in the green trace of Figure 7B. Similar observations are made when cell cultures are treated with analog 11 (Figure S6), albeit with a less pronounced phenotype, which is in agreement with the lower affinity of the analog for BfrB and correspondingly higher IC₅₀. These findings, which indicate that the analogs elicit the anticipated pyoverdin hyper-production phenotype in P. aeruginosa, suggest that they bind BfrB in the P. aeruginosa cytosol, block the BfrB−Bfd interaction, and inhibit iron mobilization from BfrB.

To obtain additional evidence that 16 blocks the BfrB−Bfd interaction and inhibits iron mobilization from BfrB in the P. aeruginosa cytosol, we resorted to visualizing BfrB-iron in native PAGE gels stained with Ferene S. A similar approach has been used to demonstrate that the Δbfd mutant irreversibly accumulates iron in BfrB. To this end, cells cultured in M63 media supplemented with 4 μM iron were treated with 16 (125 μM) or with an equivalent volume of DMSO (control). At different time points cells were harvested by centrifugation after a small aliquot had been sampled to enumerate cell density (CFU/mL). The growth curves (Figure 8A) show that at every time point the number of viable cells in the untreated cultures is approximately 2.5-fold larger than in cultures treated with 16. To visualize iron stored in BfrB, the cells harvested at different time points were lysed and the clarified supernatants were loaded onto native PAGE gels for separation and subsequent staining with Ferene S (Figure 8B); recombinant BfrB mineralized with an iron core of approximately 400 iron ions was used as a standard for the electrophoretic mobility of BfrB. Lanes loaded with lysate from untreated cells (DMSO in Figure 8B), in order to account for the nearly 2-fold larger CFU/mL observed at each
time point relative to the treated culture. In contrast, the lanes loaded with lysates obtained from cultures treated with 16 show only iron accumulation in BfrB. The distinct trends of iron accumulation in BfrB observed with the treated vs untreated (0.5x DMSO) cultures can be readily visualized in the plot of Figure 8C, which was constructed with the aid of densitometry analysis of the gel bands. It is evident that the untreated cells store iron in BfrB during the logarithmic growth phase and then mobilize the stored reserves during the stationary phase. In contrast, when cells are treated with 16, the flow of iron into BfrB appears to be mostly unidirectional, with much slower (inhibited) mobilization of iron from BfrB. Consistent with the nearly irreversible accumulation of iron in BfrB when cultures are treated with 16, measurements of total cellular iron levels normalized to viable cell counts show that P. aeruginosa cells harbor approximately twice as much iron in the treated cultures relative to the untreated control (Figure 8D). Taken together, these observations strongly support the idea that blockade of the BfrB–Bfd interaction by 16 inhibits iron mobilization from BfrB and leads to nearly an irreversible accumulation of unusable iron in the bacterial cell.

### 4-Aminoisoindoline-1,3-dione derivatives enhance the killing activity of fluoroquinolones.

Previous studies demonstrated that intact iron homeostasis is essential for bacterial cell survival under antibiotic stress, which suggests that bacterial iron homeostasis may be a potential target for boosting the action of antibiotics. Consequently, we asked if the 4-aminoisoindoline-1,3-dione probes developed to disrupt bacterial iron homeostasis by blocking the BfrB–Bfd interaction would also potentiate the killing activity of antibiotics. The idea was initially tested by treating P. aeruginosa PAO1 cultures with (i) analog 16, (ii) ciprofloxacin at the reported MIC45 (0.25 μg/mL), and (iii) a combination of ciprofloxacin and analog 16. The effect was evaluated 18 h post treatment by plating, enumerating viable cells (CFU/mL), and comparing the results to untreated cells (Figure 9A). Cultures treated with only analog 16 (125 μM) experienced the anticipated ~30% survival, and cultures treated with only ciprofloxacin experienced approximately 10% survival relative to untreated control. In comparison, cultures treated with a combination of ciprofloxacin and analog 16 experienced significantly lower survival, in an analog-concentration dependent manner, such that when analog 16 was present at 125 μM the % survival was ~50-fold lower relative to treatment with ciprofloxacin alone. Similar experiments carried out with the fluoroquinolones levofloxacin and norfloxacin revealed a similar enhancement of bactericidal activity, with approximately 50-fold lower cell survival of cultures treated with a combination of 16 (125 μM) and fluoroquinolone, relative to treatment with only the fluoroquinolone. Related experiments conducted with tobramycin and gentamycin (protein synthesis inhibitors) and with ceftazidime and imipenem (cell wall biosynthesis inhibitors) showed no enhancement of the antibacterial activity of these inhibitors when used in combination with the small molecule inhibitors of the BfrB–Bfd interaction (data not shown).

To extend these observations to include additional strains of P. aeruginosa, we conducted similar investigations with two cystic fibrosis clinical isolates (MR3B and MR60) obtained from Seattle Children’s Research Foundation. Observations made with strain MR3B (Figure 9D) are similar to those made with strain PAO1 in that cultures of M3B treated with analog 16 (125 μM) experienced ca. 50% survival and cultures treated with ciprofloxacin (0.2 μg/mL) experienced ca. 10% survival. In comparison, cultures treated with a combination of analog 16 and ciprofloxacin experienced 0.5% survival, or approximately 20-fold lower survival relative to treatment with ciprofloxacin alone. Strain MR60 is significantly more resistant to ciprofloxacin. When treated with ciprofloxacin at a concentration of 1 μg/mL, which is 4–5-fold higher than the dose used in experiments with strain PAO1 or MR3B, the MR60 strain experienced ca. 90% survival (Figure 9E). In comparison, MR60 cultures treated with analog 16 (125 μM) experienced ca. 40% survival and cultures treated with a combination of analog 16 and ciprofloxacin experienced ca. 10% survival, or approximately 4-fold lower survival relative to treatment with ciprofloxacin alone. These findings indicate that analogs of 4-aminoisoindoline-1,3-dione, such as analog 16, have the potential for inhibiting the BfrB–Bfd interaction and enhance the activity of fluoroquinolones in a variety of P. aeruginosa strains.

### DISCUSSION

Iron metabolism is emerging as an important unconventional target for the development of antibacterial strategies. The essentiality of iron for most pathogens, together with innate immune defenses which function to maintain very low concentrations of free iron in vivo (~10−20 M), present a formidable challenge to host colonization by pathogens and suggest that dysregulation of iron homeostasis constitutes a significant bacterial vulnerability. In agreement, gallium has been shown to disrupt bacterial iron metabolism, and a recent report showed that systemic gallium treatment improves lung function in patients with chronic P. aeruginosa infection. Ga3+, which has an ionic radius similar to that of Fe3+, is thought to perturb iron homeostasis by replacing Fe3+ in vital iron-utilizing proteins. Since Ga3+ cannot be reduced under physiological conditions, iron-utilizing proteins become inhibited, adversely affecting important metabolic paths. These observations, which underscore the significance of targeting iron metabolism as a viable approach to treat infections, also highlight the importance of developing rational means to dysregulate bacterial iron homeostasis to validate new targets and implement new strategies to develop novel antimicrobial therapies.

Previous investigations with P. aeruginosa showed that bacterial iron homeostasis can be perturbed by specifically interfering with the process of iron storage/mobilization from bacterioferritin. Encouraged by these results we pursued a systematic, iterative strategy based on fragment screening, structural characterization of fragment binding, and synthetic elaboration of fragment hits to discover inhibitors of the BfrB–Bfd protein–protein interaction (Table 1). These small molecule analogs of 4-aminoisoindoline-1,3-dione selectively bind BfrB at the Bfd binding site and engage pockets on the BfrB surface where Y2 and L5 from Bfd anchor. X-ray crystallographic studies showed that all analogs in Table 1 bind at the Bfd-binding site on BfrB with nearly identical poses and interactions. These observations, which underscore the selectivity of the analogs for the Bfd-binding site on the BfrB surface, validate the structure-guided approach that led to their identification as inhibitors of the protein–protein interaction. The binding selectivity of the analogs for the Bfd binding site on BfrB endows analogs such as 16 with their ability to bind BfrB in the P. aeruginosa cytosol, perturb its interaction with Bfd, and inhibit the mobilization of BfrB-iron. Consequently,
the observations reported herein constitute proof of concept for the usefulness of chemical probes designed to perturb iron homeostasis by rationally interfering with a specific protein–protein interaction in the bacterial cell. Blockade of the BfrB–Bfd interaction with these chemical probes inhibits iron mobilization from BfrB and establishes a nearly unidirectional flow of iron into BfrB, which causes a significant fraction of the cellular iron to be “trapped” in BfrB and, therefore, accumulate as an unusable resource for the cell. The nearly irreversible accumulation of iron in BfrB is probably accompanied by a depletion of free iron in the cytosol, similar to that observed with the ΔbfrD mutant, which, as expected, is manifested in a pyoverdin hyperproduction phenotype. In this context, the growth defect elicited by the inhibitors is probably related to an intracellular iron limitation induced by blockade of the BfrB–Bfd interaction, which in turn is likely to exert an inhibitory effect on the biosynthesis and repair of iron-dependent enzymes that function in central physiological processes. Hence, the chemical probes reported herein provide a valuable starting point for future studies of structure–activity relationships that could lead to the identification of high-affinity analogs as potential therapeutic lead compounds.

An important feature of utilizing chemical probes for dissecting biological systems is that these can be used alone, or in combination with other synergistic or antagonistic probes. Previous studies have shown that some antibiotics disrupt bacterial iron homeostasis and that the iron homeostasis machinery is important for bacterial cell survival in the presence of antibiotics. Given that our results show that the inhibitors of the BfrB–Bfd interaction dysregulate iron homeostasis, we asked if the inhibitors would also boost the activity of antibiotics. This idea was tested initially with ciprofloxacin, and then with two other fluoroquinolones, norfloxacin and levofloxacin. The results show that the small molecule inhibitors of the BfrB–Bfd interaction boost the bactericidal activity of the fluoroquinolones approximately 50-fold. Additional work is clearly required to understand the reasons behind these observations. It is tempting, however, to speculate that the enhancement of the killing activity brought by the inhibitors of the BfrB–Bfd interaction may be related to the intracellular iron depletion caused by inhibiting the mobilization of iron from BfrB, which limits the pool of iron required to support the biogenesis or the repair of iron-dependent enzymes. In this context, it has been proposed that bactericidal antibiotics have well-established mechanisms of action, but that, in addition to these distinct mechanisms, subsequent metabolic changes such as elevated concentrations of TCA metabolites, active breakdown of the metabolic pool, and an elevated redox state also contribute to defining bactericidal activity. It is therefore possible that the intracellular limitation of iron caused by inhibition of the BfrB–Bfd interaction impairs the biogenesis or the repair of important enzymes such as aconitase and succinate dehydrogenase of the TCA cycle, thus decreasing cell fitness and increasing the bacterial cell susceptibility to fluoroquinolone antibiotics. Consequently, it is possible to conceive a potential therapeutic strategy where inhibitors of the BfrB–Bfd interaction are used in combination with existing fluoroquinolone antibiotics.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/jacs.9b00394.

Figures S1–S6, crystallographic data (Table S1), synthetic procedures, fragment library screening using NMR spectroscopy, measurement of dissociation constants, and 1H and 13C spectra (PDF)

Accession Codes

Coordinates and structure factors were deposited to the Worldwide Protein Databank (www.wwPDB.org) with accession codes: Apo-BfrB (6NLF); C895/K96C BfrB-fragment 1 (6NLG); BfrB-analog 11 (6NLI); Apo-BfrB-analog 12 (6NLJ); BfrB-analog 13 (6NLK); BfrB-analog 14 (6NLL); BfrB-analog 15 (6NLM); BfrB-analog 16 (6NLN).

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Notes

The authors declare no competing financial interest.

ACKNOWLEDGMENTS

This study was supported by a grant from the National Institutes of Health to M.R. (AI125529). M.R. also thanks the National Science Foundation for support (MCB1615767). Use of IMCA-CAT beamline 17-ID at the Advanced Photon Source was supported by the companies of the Industrial Macromolecular Crystallography Association through a contract with the Hauptman–Woodward Medical Research Institute. Use of the Advanced Photon Source was supported by the U.S. Department of Energy, Office of Science, Office of Basic Energy Sciences, under Contract No. DE-AC02-06CH11357. Use of the University of Kansas Protein Structure Laboratory was supported by a grant from the National Institute of General Medical Sciences (P30 GM110761) at the National Institutes of Health. P. aeruginosa strains MR3B and MR60 were provided by Seattle Children’s Research Foundation, supported by NIH Grant P30DK089507.

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