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Small Molecule Antivirulents Targeting the Iron-Regulated Heme Oxygenase (HemO) of *P. aeruginosa*

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Abstract

Bacteria require iron for survival and virulence and employ several mechanisms including utilization of the host heme containing proteins. The final step in releasing iron is the oxidative cleavage of heme by HemO. A recent computer aided drug design (CADD) study identified several inhibitors of the bacterial HemOs. Herein we report the near complete HN, N, CO Cα and Cβ chemical shift assignment of the *P. aeruginosa* HemO in the absence and presence of inhibitors (*E*)-3-(4-(Phenylamino)phenylcarbamoyl) acrylic acid (**3**) and (*E*)-*N*-(4-(dimethylamino)benzylidene) diazenecarboximidhydrazide (**5**). The NMR data confirms the inhibitors bind within the heme pocket of HemO consistent with *in silico* molecular dynamic simulations. Both inhibitors and the phenoxy derivative of **3** have activity against *P. aeruginosa* clinical isolates. Furthermore **5** showed antimicrobial activity in the *in vivo* *C. elegans* curing assay. Thus targeting virulence mechanisms required within the host is a viable antimicrobial strategy for the development of novel antivirulents.

P. aeruginosa is a leading cause of nosocomial infection in intensive care patients, neutropenic patients, and in respiratory infections of cystic fibrosis patients.^{1,2} Additionally, *P. aeruginosa* is naturally resistant to many antibiotics. This natural resistance combined with the rapid rise in multi-drug resistant strains has led to a significant healthcare crisis.³⁻⁵ As a result of the rise in multi-drug resistant strains of *P. aeruginosa* there is an increasing need to identify new therapeutic targets while simultaneously reducing the emergence of drug resistance.

The acquisition of iron is essential for the survival, growth and virulence of bacterial pathogens. Gram negative bacteria acquire iron via receptor mediated iron-siderophore scavenging mechanisms and in many pathogenic strains via analogous receptor systems specific for heme and heme proteins.^{6,7} Furthermore, heme has been shown to be the preferred source of iron during infection in a number of bacterial pathogens including *Staphylococcus aureus*⁸ and *Bordetella pertussis*.⁹ The opportunistic gram negative pathogen *P. aeruginosa* encodes two inter-dependent heme uptake systems, the *Pseudomonas* heme utilization (*phu*) and heme assimilation system (*has*).¹⁰ The *phu* uptake system is comprised of the outer-membrane receptor, PhuR, and a periplasmic transport system comprising PhuT, a soluble receptor for the ATP-dependent permease (ABC-transporter), PhuUV and the cytoplasmic heme binding protein, PhuS. In contrast, the *has* system encodes the soluble excreted hemophore (HasA) and a TonB-dependent outer-

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BioMagResBank ID Codes: The ¹H, ¹³C and ¹⁵N chemical shifts were deposited with the BioMagResBank (<http://www.bmrb.wisc.edu>) under the BMRB accession number 18988.

membrane receptor (HasR).^{11,12} The *has* operon in contrast to the *phu* system lacks the periplasmic uptake genes and is presumed to internalize heme via the PhuUV/T ABC-transporter.¹³ The final step in heme utilization is the degradation of heme to iron, δ and β -biliverdin and CO by heme oxygenase (HemO).¹⁴

As the ability to utilize heme as an iron source is required for virulence but not survival outside of the host-pathogen interaction, we hypothesized that heme utilization would provide a novel therapeutic target with less selective pressure to undergo mutagenesis leading to resistance. As the final step in the release of iron from exogenously acquired heme, HemO is critical and hence represents a potential novel therapeutic target. In an earlier computer aided drug design (CADD) study we identified a series of small molecule inhibitors of the HemOs from *P. aeruginosa* and *N. meningitidis*.¹⁵ In the current report we have employed STD and chemical shift NMR analyses to determine both the inhibitor binding epitope and the site of interaction on HemO, respectively. Independent MD simulations and docking experiments are consistent with the NMR data where the inhibitors bind to specific sites within the heme pocket. Furthermore, consistent with the *in vitro* analysis the inhibitors were shown to have biological activity against *P. aeruginosa* clinical isolates and in a *C. elegans* curing assay.

Results

Binding affinity of inhibitors to the *P. aeruginosa* apo-HemO

In a previous CADD study of potential HemO inhibitors we identified **3** and **5**.¹⁵ The binding affinity (K_D) of **3** and **5** against the *P. aeruginosa* HemO (previously termed pa-HO) were reported to be in the range 20-30 μ M and both inhibitors were further shown to inhibit biliverdin production in an *E. coli*-*N. meningitidis* HemO expression system. In the current studies we further characterize the binding of the previously characterized **3**, as well as **4** the phenoxy derivative of **3** to the *P. aeruginosa* HemO. The binding affinity (K_D) of **4** is similar to that previously reported and determined herein for **3** (Fig 1 and Table 1). In the remainder of the manuscript all reference to HemO relates to the *P. aeruginosa* enzyme.

STD-NMR of inhibitors

STD-NMR was used to further define the binding epitope of **3** and **5** to HemO. As shown in Fig 2A saturation transfer from the protein to all detectable protons on **3** is observed. The relative intensities of the peaks observed in the difference spectrum is dependent on both the longitudinal relaxation times T1 in the free state and the proximity of the proton to the protein. Integration of the peaks in the 10 sec delay spectrum compared with those of reference spectrum (Fig 2B) indicates the protons receiving the largest relative saturation transfer.²⁶ The proton receiving the largest saturation transfer in the difference spectrum for each inhibitor was set at 100%. For **3** the peak area of proton H_a in the difference spectrum, was assigned arbitrarily as 100 percent, and the remaining peak areas are reported relative to proton H_a, considering adjustment for the number of protons representing a peak (Fig 2B and C). Similarly for **5** the peak area of proton H_b in the difference spectrum was assigned arbitrarily as 100 percent, and peak areas of the remaining protons were calculated relative to H_b. Therefore the protons of **3** in closest proximity to the protein are those of the phenyl rings, with the acrylic acid side chain having a weaker interaction (Fig 2C). Similarly, the STD-NMR of **5** (Fig. 2D) indicates that the protons receiving the largest saturation transfer compared with the reference spectrum (Fig 2E) are those of the benzene ring and the benzylic proton. The proton signal of the dimethylamino group is relatively weak. The low level of the saturation transfer might due to the free rotation of the dimethylamino group along the C-N bond in the active site (Fig. 2F), which limits the interaction of either methyl group to the protein.

NMR assignment and chemical shift mapping of HemO

The ^1H - ^{15}N HSQC spectrum of apo-HemO shows well-dispersed peaks indicative of a folded protein (Fig S1 of the Supporting Information). Addition of saturating concentrations of **3** or **5** resulted in significant shifts in specific resonances (Fig S1).

For the apoprotein, backbone chemical shifts (N, NH, CA, CB, CO) were assigned for 171 out of 198 residues. Among the assigned residues, the amide protons of 16 residues were not assigned; however, their corresponding CA, CB or CO resonances were detected from the respective $i+1$ amide resonances in the HNCOC, HNCACB or HNCO spectra.

Specifically, CA, CB and CO were assigned for; Thr-9, Gln-11, Pro-38, Arg-42, His-57, Pro-61, Asp-81, Pro-96, Gln-100, Ser-122, Ala-155, CA and CO were assigned for Pro-38, Gly-74, Gly-152 and F186, and CO were assigned for Phe-130 and His-146. The following 30 residues in apo-HemO were not assigned: Met-1, Asp-2, Pro-6, Glu-7, Ser-8; Asn-23, Glu-24, Pro-25; His-26; Gln-27, Arg-28, Glu-30, Ser-31, Val-33, Lys-34, Pro-73, Pro-94, Val-95, Leu-124, Gly-125, Ala-126, Ala-127, Phe-128, Leu-129, Gly-143, Ala-144, Arg-145, Pro-150, Glu-151 and Arg-154, presumably due to resonance overlap from fast exchange of the amide proton with bulk water or other residues.

On the basis of the resonance assignments of HemO chemical shift perturbations were performed for HemO in complex with **3**. The resonance assignments were based on the assignment of the apo-HemO and confirmed with data from 3D HNCA and 3D ^{15}N -resolved HSQC-NOESY; 163 residues were assigned with backbone shifts; among these, 9 residues were assigned with CA, CB or CO but no amide: Pro-38, Arg-42, His-57, Pro-61, Gly-74, Pro-96, Gln-100, Phe-130, and Ala-155. The following 35 residues were not assigned: Met-1, Asp-2, Pro-6, Glu-7, Ser-8, Thr-9, Arg-10, Gln-11, Asn-23, Glu-24, Pro-25, His-26, Gln-27, Arg-28, Pro-73, Asp-81, Asp-87, Pro-94, Val-95, Leu-116, Ser-122; Lys-123, Leu-124, Gly-125, Ala-126, Ala-127, Phe-128, Leu-129, Lys-132, Arg-145, His-146, Pro-150, Glu-151, Gly-152, and Arg-154. Interestingly several of the residues not assigned in the inhibitor **3** bound complex (Thr-9, Arg-10, Gln-11, Asp-81, Asp-87, Leu-116, Ser-122, Lys-123, Lys-132, His-146 and Gly-152) were previously assigned in the apo-HemO. Similarly, several residues assigned only in the inhibitor bound HemO were not assigned in the apo form (Glu-30, Ser-31, Val-33, Lys-34, Gly-143 and Ala-144). In addition, the amide resonance of Phe-186 was not observed in the free protein, but was observed and assigned in the HemO-inhibitor **3** complex. Similarly, residues previously observed in the apo structure that were not assigned on titration with **5** mirrored the profile for **3** with the exception of Asp-87 and Leu-116 which were assigned in the apo and inhibitor bound protein. However, in contrast to **3**, titration with **5** did not allow assignment of the proximal helix residues (Glu-30, Ser-31, Val-33 or Lys-34) but rather only the distal residue Ala-144. The chemical shift data for the apo-HemO and inhibitor complexes were deposited with the BioMagResBank (<http://www.bmrb.wisc.edu>) under the BMRB accession number 18988.

Chemical shifts monitored as a function of concentration of **3** fall into three categories: i) residues not assigned in the apo-HemO that increased in intensity in the presence of the inhibitor; ii) chemical shifts on increasing inhibitor concentration; and iii) resonances detected in the apo-HemO that are lost on complex formation with **3**. The magnitude of chemical shift changes are shown in Fig 3A. The residues visible in the HSQC experiments only in the inhibitor bound HemO, Glu-30, Ser-31, Val-33 and Lys-34 are located on the proximal helix along with Phe-186 from the adjacent C-terminal helix (Fig 3B). The appearance of these residues suggests that the inhibitor is binding and stabilizing this region of the heme pocket. A second set of peaks showing the greatest chemical shifts on titration with **3**, including Ser-35, Lys-36, Phe-39, Asn-187 and Asp-191, are also located within the heme binding pocket (Fig 3B). In the backside of the heme pocket Phe-55 and Phe-186 are

situated within a hydrophobic cavity and show significant chemical shift perturbation on titration with **3**. Taken together the chemical shift data is consistent with the initial database screening of HemO inhibitors where several potential inhibitors (including **3** and **5**) were predicted to have extensive interactions with the proximal helix and within a hydrophobic cavity at the back of the heme binding site. In addition to the appearance of resonances on titration with **3** several residues could be assigned only in the apo-HemO and were most notably in the distal helix including residues Ser-122 and Lys-132 (Fig 3B).

Chemical shift perturbations as a function of inhibitor concentration within the heme binding pocket show 1:1 stoichiometry suggesting **3** binds within the heme pocket through interactions with the proximal helix (Fig 5B). Although chemical shift perturbations were observed for residues distant from the binding site these most likely arise from the fact they are located on the C-terminal surface helix within 3-5 Å of the proximal helix residues.

Inhibitor **5** was also shown to perturb residues within the heme binding pocket. However, in contrast to **3** the chemical shifts mapped primarily to residues on the distal helix including Leu-106, Ser-108, Glu-111, Gly-114, Trp-115, L116, Phe-117, Val-118, Ser-119 and Gly-121 (Fig 4A). Interestingly, Phe-117, Val-118, Gly-121 and Ala-185 located within heme binding pocket are close to the protein surface (Fig 4B). Ala-185 sits in the heme binding pocket in close proximity to Arg-188 of the C-terminal helix with these two residues showing the largest chemical shifts. Ala-185 is also in close proximity to the side chain of Trp-115 and a cluster of distal helix residues with significant chemical shifts including Leu-106, Ser-108, Glu-111, and Gly-114. The side chain of Arg-188 extends out to the surface where it is in close proximity to several residues within a surface exposed loop including Gly-98, Asp-99, Val-102, Glu-104 and Leu-107 (Fig 4C).

The magnitude of the chemical shifts on increasing concentrations of **5** fall into two classes: i) those residues within the distal heme binding pocket that saturate at 1:1 molar equivalents (Trp-115, Val-118 and Gly121); and ii) those that show no saturation effect on binding and are located on a flexible surface loop or the C-terminus helix (Fig 5C). Interestingly, residues in the C-terminal helix and the flexible surface loop are within 3-5 Å of the heme binding pocket and were shown to have measurable chemical shifts on titration with either **3** or **5**. Based on the non-saturable nature of the chemical shifts and their surface location and conformational flexibility we propose the shifts are a direct result of their proximity to residues within the heme binding pocket that directly contact the inhibitor. Although we cannot rule out a second binding site on the surface of the protein the current chemical shift data and previous biochemical studies where the inhibitors were shown to have 1:1 stoichiometry, and inhibit the production of biliverdin in an *in vivo* *E. coli* expression assay, support **3** and **5** binding to distinct sites within the heme pocket.¹⁵ This hypothesis is further supported by the *in silico* molecular dynamic simulations in the following section.

***In silico* molecular dynamic (MD) simulations and docking calculations**

In order to carry out the MD simulations it was necessary to obtain conformations of the apo form of HemO in which the heme binding pocket was in an “open” or accessible state. These were obtained via a MD simulation of the apo protein initiated from the crystal structure of HemO (as outlined in the Experimental Section). To identify open conformations, the accessibility of the heme binding pocket was monitored by following the proximal His-26 to Gly-121 distance as a function of time (Fig S2). From this plot, the more accessible conformations of the binding pocket are sampled at the 11.38, 14.74, 52.00 and 75.92 ns snapshots. These four conformations of apo-HemO were selected for the docking calculations of both **3** and **5**. As shown in Fig 6 the top three docked poses against each conformation of **3** are consistent with the chemical shift perturbations where the inhibitor

spans the heme binding pocket taking advantage of several interactions along the proximal helix. These interactions include several residues that are assigned only in the inhibitor bound form, namely Glu-30, Ser-31, Val-33 Lys-34 and Phe-186, while those showing the greatest chemical shifts are Ser-35, Lys-36, Phe-55 and Phe-39. Distances of the phenyl ring and acrylic acid side chain of **3** with the center of mass of the sidechain NZ of Lys-34 and phenyl ring of Phe-55 for each pose and snapshot are shown in Table 2. The convergence of distances for the terminal phenyl ring of **3** with Phe-55 is consistent with a model whereby the terminal phenyl ring binds within a hydrophobic cavity toward the back of the heme binding pocket. The rest of the molecule spans the remainder of the pocket taking advantage of several interactions on the proximal helix (Fig 6). Interestingly, in all three poses and snapshots the acrylic acid side chain shows the greatest variation in conformational sampling. This is consistent with STD-NMR where the protons in closest proximity to the protein are those located on the phenyl rings with those on the acrylic acid side chain having the least interaction (Fig 2).

Inhibitor **5** was also docked to the four “open” conformations determined in the MD simulations (Fig S2). As shown in Fig 7 the top three poses bind within the heme binding pocket taking advantage of interactions with several distal helix residues in the proximity of Gly-121. The similar distances of the terminal methyl groups and the hydrazide from Phe-55 in the top three poses of **5**, and the lack of convergence of the terminal groups, is consistent with **5** binding at the surface of the heme binding pocket (Table 3). The docking studies and chemical shift perturbations of Phe-117, Val-118 and Gly-121 located close to the surface of the heme binding pocket further support a model whereby **5** binds close to the surface of the protein (Fig 5). Furthermore, the binding epitope for **5** determined by STD-NMR (Fig 2F) indicates that the primary site of interaction with the inhibitor is through the benzene ring most likely through hydrophobic interactions with the Phe-117, Val-118 and Gly-121 residues on the distal helix.

Antimicrobial activity of inhibitors

The minimum inhibitory concentration (MIC₅₀) for *P. aeruginosa* PAO1 and the clinical strains was determined for each inhibitor. The clinical strains tested were *P. aeruginosa* WR5¹⁶ a burn wound isolate and FRD1¹⁷ a mucoid cystic fibrosis isolate. As shown in Table 1 the MICs for **3**, **4** and **5** are similar for all strains tested. However, while the MICs are well above what would be termed as acceptable for antimicrobial activity there are several factors that should be considered in determining the suitability of the compounds as lead inhibitors. Firstly it is becoming increasingly more evident that *in vitro* MICs alone are not predictive of a pharmacologic response in the host.^{18,19} Therefore, such measurements of drug potency must also be accompanied by an integrative approach combining pharmacokinetics and pathogen susceptibility. *In vitro* susceptibility testing is effective when targeting metabolic pathways essential for survival irrespective of the ecological niche. However, when targeting a metabolic pathway such as extracellular heme utilization, which is required for virulence but not survival, *in vitro* MICs are less likely to be an effective measure of drug potency. We propose that such inhibitors are more correctly termed “antivirulents” and require screening in a simple host-pathogen model.

The *C. elegans* model has been utilized extensively as a screen for host pathogen interactions where direct translation of host response factors to higher organisms has been shown. Previous studies of *P. aeruginosa* infection in *C. elegans* have shown that the rate of killing depends on the composition of the media, where high osmolarity medium induces a “fast-killing” and minimal media a “slow-killing”. The “slow-killing” response involves an infection process that results in colonization and infection of the worm intestine, whereas “fast-killing” occurs via a distinct mechanism. Therefore, the “slow-killing” process

provides a model infection system that is tractable to pathogenicity of *P. aeruginosa* and host immune response factors. Furthermore, *C. elegans* provides a valuable model system for high-throughput screening of potential antimicrobials.²⁰ As shown in Fig 8A exposure of *C. elegans* to *P. aeruginosa* PAO1 results in a reduction of the average lifespan of the worm by 50% from 10 to 5 days. A significant colonization and infection of the worm intestine on exposure to *P. aeruginosa* was observed at day 4 as determined by the colony forming units (Fig 8B). In contrast worms transferred to *E. coli* plates containing 250 μ g/ml of **5** had a significant reduction in the cfu to levels close to that of the uninfected worms. The significant reduction in cfu combined with the increased lifespan of the worm confirms that **5**, despite its relatively poor *in vitro* MIC value, has significant activity in a host pathogen model.

Discussion

An important consideration in the initial CADD screen for bacterial HemO inhibitors was selectivity for the bacterial versus the human heme oxygenases HO-1 and HO-2. Comparison of the active sites of the bacterial HemOs from *N. meningitides* and *P. aeruginosa* with the human HO-1 and HO-2 showed the solvent accessible surface to be much lower (7.5 Å) in the bacterial versus the human HOs (43.6 to 59.7 Å). Such structural differences allowed for a screen of small molecule inhibitors of the bacterial HemOs that would potentially have minimal activity toward the human enzymes. Recent reports of high affinity inhibitors of the human HO-1 and HO-2 have been described.^{21,22} In contrast to the inhibitors described herein that target the apo-HemO, the nanomolar affinities for the HO-1 and HO-2 inhibitors derive from the ability of the imidazole moiety to bind directly through the heme iron. In an attempt to discriminate between the human and bacterial enzymes we have employed a strategy that targets differences in the apoprotein heme binding sites rather than targeting inhibition of the holo enzymes. The identification and characterization of **3** and **5** (Scheme 1) as inhibitors of apo-HemO¹⁵ led us to further characterize the site of interaction with HemO as well as the binding epitopes of the ligands. Preliminary STD-NMR of **3** and **5** indicated that in both cases the aromatic groups make a significant contribution to the interaction with HemO. In an effort to further define the inhibitor binding site we undertook the complete backbone assignments of HemO. Somewhat fortuitously we were also able to utilize the binding properties of **3** to identify residues that could not be assigned in the apo-HemO. Interestingly, several of the residues that were not assigned in the apo-HemO but detected on inhibitor binding are located within the heme binding pocket including Glu-30, Ser-31, Val-33, and Lys-34 on the proximal helix and Phe-186 from the adjacent helix (Fig 3B). The dynamic behavior of the proximal helix in the absence of the heme ligand is well documented for the human HO-1.^{23,24} Furthermore, the decreased freedom in the proximal helix on inhibitor binding is propagated out to the surrounding residues Ser-35, Lys-36, Phe-39, Phe-55, Asn-187 and Asp-191 that define the inhibitor binding site of **3** (Fig 3A and B). Concomitant with the reduction in conformational freedom of the proximal helix on inhibitor binding resonances on the distal face and connecting loops were observed to disappear. Interestingly, the distal helix Lys-132 which participates in a key interaction with the heme propionate stabilizing the orientation of heme within the protein becomes disordered on binding **3**.²⁵ This is in contrast to Lys-34 that stabilizes the alternate heme propionate through electrostatic interactions and is only detected on titration with inhibitor **3**. The chemical shift perturbations are consistent with inhibitor **3** primarily binding through interactions with residues on the proximal heme binding face.

The chemical shift map of **5** indicates that in contrast to **3**, where the inhibitor has extensive contact shifts in the proximal helix, the chemical shifts observed for **5** map to the distal helix, specifically residues Phe-117, Val-118, Gly-121 and Ala-185 of the C-terminal helix (Fig 4A and B). Several residues on the C-terminal helix and flexible surface loop on

binding **3** or **5** give chemical shifts that do not show concentration dependent saturation. This is more pronounced with **5** where we attribute this effect to either allosteric effects of inhibitor binding within the pocket or a surface site on the backside of the protein away from heme binding pocket (Fig 5C). However, as we have previously reported **3** and **5** bind to HemO with a 1:1 stoichiometry and inhibit the production of biliverdin in an *E. coli* over-expression assay.¹⁵ Therefore, we conclude that the primary binding site of both **3** and **5** lies within the heme binding pocket. Furthermore, while **3** and **5** show similar biological activity the chemical shift data indicates they bind to distinct regions on the proximal and distal face of the heme pocket, respectively. The significant shift in the amide protons observed in the HSQC on titration of HemO with **3** or **5** (Fig S1) is not due to changes in pH and is attributed to a combination of direct binding interactions with the inhibitor and longer range allosteric effects on inhibitor binding.

The binding site and orientation of both **3** and **5** is further supported by the molecular docking studies whereby the top three poses for each inhibitor are consistent with binding at the proximal and distal helices, respectively. For both inhibitors the STD-NMR and docking calculations confirm that the aromatic groups play a major role in binding and orienting the inhibitor within the heme binding pocket. Interestingly, docking of **3** places the terminal phenyl ring toward the back of the binding pocket where it can interact through π -stacking interactions with Phe-55. Consistent with the molecular docking indicating the acrylic acid side chain to have significant conformational sampling the detectable acrylic acid protons are not in close contact with the protein as determined by STD-NMR. Therefore, there is conformational flexibility in the binding mode of the acrylic acid side chain where it most likely faces out toward the surface of the protein and is partially stabilized via interaction with Lys-34 (Fig 4C and 6).

Similarly, the STD-NMR of **5** shows the benzene ring and neighboring benzylic proton in close proximity to the protein. The chemical shift perturbation and docking studies are consistent with the aromatic ring interacting with a hydrophobic patch of residues including Phe-117, Val-118 and Gly-121 of the distal helix (Figs 5B and 7). Interestingly the distal residues are located close to the protein surface on the distal face of the heme binding pocket (Fig 5B). Although we have no definitive evidence of how or where the terminal carboximidhydrazide group is interacting the docking calculations indicate that this region along with the terminal dimethylamino do not bind at a specific site as judged by the lack of convergence of distances from the buried Phe-55 (Table 3). The data is consistent with STD-NMR where the benzene ring is in close proximity to the protein and provides the anchor to the hydrophobic residues on the distal face of the pocket.

Despite the alternate binding sites within the heme pocket both **3** and **5** were both previously shown to inhibit enzyme activity.¹⁵ The inhibition of HemO activity further translates to antimicrobial activity as **3**, **4** and **5** all inhibit the growth of several clinical isolates of *P. aeruginosa*. However, while the MIC values for the HemO inhibitors are relatively weak we believe this is a result of the inability of MIC assays to determine antivirulence versus antimicrobial activities (Table 1). This is further supported by our previous studies that show growth inhibition in liquid culture of *P. aeruginosa* in the presence of **3** and **5** is solely heme dependent and can be easily overcome on addition of non-heme iron.¹⁵ Therefore, we performed a more accurate and semi-high throughput measure of antivirulence using the *C. elegans* host pathogen model. The *C. elegans* curing assay has been used successfully to screen for inhibitors of *E. faecalis* where several compounds active in the *in vivo* *C. elegans* curing assay were shown to have no inhibitory activity *in vitro* MIC assays.²⁰ The authors proposed that the discrepancy was the result of the ability to select for molecules that target virulence factors required for infection in the *C. elegans* model that would otherwise be masked in the *in vitro* MIC assays. Consistent with this hypothesis *P. aeruginosa* infected

worms when transferred to plates containing **5** at the MIC₅₀ level (250 µg/ml) were shown to completely eradicate the *P. aeruginosa* infection and rescue the lifespan of the infected worms to that of the uninfected worms (Fig 8). As the inhibitor has to be absorbed by the worm and then be actively taken up by the bacteria on reaching the site of infection we conclude that **5** is active at concentrations well below the calculated MIC₅₀ based on the complete eradication of the infection.

In summary we have determined binding modes of two lead inhibitors of the iron-regulated HemO the final step in the heme utilization by the opportunistic pathogen of *P. aeruginosa*. Both binding modes independently inhibit enzyme activity and lead to reduced virulence of *P. aeruginosa* clinical strains that can be further exploited in the development of inhibitors that simultaneously target both sites. A fragment based drug design approach selecting for inhibition at both sites has the potential to lead to the development of inhibitors with increased selectivity and potency for the bacterial HemOs while limiting interaction with the human HOs. Based on our preliminary findings future CADD and SAR studies utilizing NMR and crystallographic methods combined with *in vivo* analysis in the *C. elegans* curing assay provides the foundation for the development of selective bacterial HemO inhibitors. Such novel approaches targeting mechanisms required for virulence but not survival may lead to the development of antivirulants that reduce the pressure to undergo mutations that lead to resistance. The development of novel agents is becoming increasingly more urgent with the rapid rise in drug resistant *P. aeruginosa* infections within the hospital and community settings.

Experimental Section

Nematode and bacterial strain media and growth conditions

C. elegans glp-4(bn2)I was maintained by standard methods as described.²⁶ Worms were maintained on Nematode Growth Medium (NGM) (LabScientific, Livingston, NJ) with *E. coli* OP50 as a food source. Killing and rescue assays were performed on PGS agar (1% Bacto-Peptone, 1% NaCl, 1% Glucose, 0.15 M Sorbitol, and 17% Bacto-Agar). Bacteria were routinely grown in Luria-Bertani (LB) broth at 37 °C at 200 rpm. *P. aeruginosa* and *E. coli* strains were maintained on *Pseudomonas* Isolation Agar (PIA) (Becton Dickinson, Franklin Lakes, NJ) and LB-plates, respectively. When necessary the following antibiotic concentrations were used: for *E. coli*, 100 µg/ml ampicillin; for *P. aeruginosa*, 200 µg/ml gentamicin. The *hemO* gene in pET21a was transformed into *E. coli* strain BL21 (DE3) *pLysS* [F-*ompT hsdSB* (rB-mB-) *gal dcm* (DE3)] for protein expression as previously described.¹⁴

Growth inhibition of *Pseudomonas aeruginosa* strains in the presence of apo-HemO inhibitors

Growth inhibition assays were performed as previously described with slight modification. Briefly, a culture (20 ml) of each strain was grown from a single colony for 8 hrs at 37 °C in LB media and diluted back to an OD₆₀₀ of 0.05. A 96-well plate assay was set up with 200 l of the respective cultures in LB-media either alone or containing 250-1500 M compound. The OD₆₀₀ for all wells was recorded on a SpectraMax Plus 96-well plate reader (Molecular Devices) at 6, 12 and 24 hours. To assure no absorbance contributions from the inhibitors the change in OD₆₀₀ was determined on subtraction of the absorbance from the blank containing the equivalent concentration of inhibitor and 200 µl gentamicin to prevent *E. coli* growth. The MICs calculated as previously described were an average of three separate experiments.

C. elegans killing and rescue

C. elegans glp-4(bn2) worms were synchronized by collecting eggs from gravid adult worms and hatching the eggs overnight in M9 media. L₁-stage worms were plated on NGM agar and grown for 8-12 hours at 25°C after which they were harvested and washed in M9 buffer. Killing assays were performed by seeding 30 young adult worms on a lawn of *P. aeruginosa* PAO1 on PGS-agar for 12 hours. Worms were washed in M9 buffer and reseeded on a lawn of *E. coli* OP50. Plates were examined daily for worm viability and worms were considered dead when they no longer responded to touch. Rescue experiments were performed as described above except the worms were reseeded on *E. coli* OP50 PGS-agar plates containing 200 μ M of **3**. The data represents an average of three experiments.

The level of infection was determined by calculating the bacterial colony forming units recovered from the worm intestines at days 24, 48, 72 and 96 hours post infection as previously described.²⁰ Briefly, ten live worms were collected from negative control plates (worms fed only *E. coli*), positive control plates (worms exposed to *P. aeruginosa* no treatment) and worms exposed to *P. aeruginosa* and treated with **3**. The worms were washed three times in M9 buffer containing 1 mM sodium azide (250 μ l) and 50 μ l of buffer was removed to determine the external cfu. Silicon carbide beads 1.0-mm in size (Biospec Products, Bartlesville, OK) were added to the worms and the remaining 200 μ l was vortexed at high speed for one minute. The cell debris was pelleted and an aliquot (50 μ l) was serially diluted and plated on PIA plates for cfu determination.

Protein Purification

P. aeruginosa HemO was purified by a slight modification of the previously described procedure.¹⁴ Residual biliverdin was removed by binding to Q-Sepharose (1 cm $\times$ 20 cm) equilibrated in 10 mM diaminopropane (pH 9.0). The apoprotein was eluted with a 0-250 mM KCl gradient in the same buffer and the biliverdin was retained on the columns. Apo-HemO peak fractions were pooled and dialysed against 20 mM Tris-HCl (pH 7.5).

Chemical synthesis of (E)-3-(4-(Phenylamino)phenylcarbamoyl)acrylic acid (**3**) and (E)-3-(4-Phenoxyphenylcarbamoyl)acrylic acid (**4**)

As shown in Scheme 1.0 inhibitors **3** and **4** were synthesized in excellent yields by an aminolysis of maleic anhydride using commercially available aniline derivatives **1** and **2**, respectively.²⁷

(E)-3-(4-(Phenylamino)phenylcarbamoyl)acrylic acid (**3**)

To a solution of **1** (0.184 g, 1.0 mmol) in EtOAc (2 mL) was added maleic anhydride (98 mg, 1.0 mM). The reaction mixture was stirred at room temperature for 3 h, and then diluted with Et₂O (20 mL). The organic layer was extracted by saturated NaHCO₃ solution, and the resulting aqueous layer was acidified with 1N HCl until precipitate formed. The aqueous mixture was extracted with EtOAc (3 $\times$ 1 mL), and the combined organic layers were dried over Na₂SO₄. The solvent was removed *in vacuo* to give compound **3** (267 mg, 0.95 mmol, 95%) as an orange powder. ¹H NMR (400 MHz, DMSO-*d*₆) δ : 13.68 (br s, 1H), 10.67 (br s, 1H), 8.14 (s, 1H), 7.50 (d, 2H, *J* = 8.4 Hz), 7.21 (t, 2H, *J* = 8.0 Hz), 7.04 (t, 4H, *J* = 8.4 Hz), 6.79 (t, 1H, *J* = 7.2 Hz), 6.44 (d, 1H, *J* = 11.6 Hz), 6.29 (d, 1H, *J* = 12.8 Hz). ¹³C NMR (100 MHz, DMSO-*d*₆) δ : 163.2, 140.2, 132.0, 131.3, 129.6, 121.4, 119.8, 117.8, 116.6. Elemental analysis for C, H, N obtained 66.95 $\pm$ 0.03, 4.99 $\pm$ 0.04 and 9.65 $\pm$ 0.01 % compared to the theoretical values of 68.07, 5.00 and 9.92 %, respectively.

(*E*)-3-(4-Phenoxyphenylcarbamoyl)acrylic acid (**4**)

Compound **4** was synthesized using a procedure similar to that of **3** (256 mg, 0.91 mmol, 91%). ¹H NMR (400 MHz, DMSO-*d*₆) δ: 13.14 (br s, 1H), 10.45 (s, 1H), 7.60 (d, 2H, *J* = 8.4 Hz), 7.34 (t, 2H, *J* = 8.0 Hz), 7.08 (t, 1H, *J* = 6.8 Hz), 6.96 (t, 4H, *J* = 10.8 Hz), 6.43 (d, 1H, *J* = 12.4 Hz), 6.26 (d, 1H, *J* = 11.6 Hz). ¹³C NMR (100 MHz, DMSO-*d*₆) δ: 167.2, 134.8, 132.1, 130.9, 130.4, 123.6, 121.7, 119.8, 118.5. Elemental analysis for C, H, N obtained 67.65 ± 0.03, 4.53 ± 0.02 and 4.8 ± 0.02 % compared to the theoretical values of 67.84, 4.63 and 4.94%, respectively.

E-*N*-(4-(dimethylamino)benzylidene) diazenecarboximidhydrazide (**5**) was previously purchased from ChemBridge Corporation, San Diego, CA.

Determination of binding affinities (*K_D*) of **3** and **4**

Fluorescence experiments were performed as previously described.¹⁵ Briefly, titrations were performed by addition of increasing concentrations of the inhibitors (0.05–500 μM) while maintaining apo-HemO protein concentrations at 1 μM in 20 mM Tris-HCl (pH 7.5). The optimal excitation wavelength for apo-HemO is at 295 nm, and the fluorescence emission was monitored from 300 to 500 nm. The dissociation constants (*K_D*) were calculated from reciprocal plots of 1/Δ*A* vs 1/[*I*] where the decrease in fluorescence, Δ*A*, at the maximum emission (330 nm) represents the fraction of occupied binding sites and [*I*] the concentration of the inhibitor. The slope of the curve equals *K_D*, as described by the following equilibrium:

All experiments are an average of three separate experiments.

NMR sample preparation

All proteins for resonance assignments were prepared in 0.5 mL of 50 mM potassium phosphate and 100 mM KCl in 95% H₂O/5% D₂O (pH 7.4), also containing 3(trimethylsilyl)propane-1,1,2,2,3,3-*d*₆-sulfonic acid, sodium salt (DSS). Proton chemical shifts are referenced to DSS. ¹⁵N chemical shifts are referenced indirectly to DSS ¹³C.

For all saturation transfer difference (STD) experiments a saturated solution of inhibitors was prepared as follows: a few grains of the compound was added to 1 mL of 50 mM sodium phosphate buffer containing 100 mM NaCl in 99.9 % D₂O (pH 7.4) and vortexed thoroughly. The sample was then allowed to sit at room temperature for about 4 hours. The saturated inhibitor solution was centrifuged for 1 min at 10,000 × *g* to remove any remaining solid material. A 600 μL of saturated inhibitor was added to 3 to 5 μL of 3 to 4 μM HemO, along with 3 μL of TSP (in a stock solution of 50 mM sodium phosphate buffer in 99.9% D₂O). The concentration of **3** was calculated from the peak areas relative to trimethylsilylpropionic acid (TSP) (2.2 mM in proton). The final concentration of the TSP peak area was used to measure the concentration of inhibitor to be 5 mM and therefore the contribution to the peak areas measured for the inhibitor from the protein (3–4 μM) were considered negligible.

NMR data collection

STD-NMR: STD experiments were performed at 25°C on a Varian INOVA 500 spectrometer, using the pulse scheme previously described without the T1ρ filter.²⁸ Solvent suppression was achieved with a 3-9-19 WATERGATE pulse sequence. The duration of the selective saturation period was 2 s, irradiation consisted of a series of 50 ms Gaussian

selective pulses, separated by 1 ms delay, at a field strength of 100 Hz. Delay between fids was set to 2 sec and 1000 to 2000 scans were collected. In order to minimize the contributions from differential relaxation rates the experiments were repeated with a 10 sec delay between scans which is four times that of the longest T1. On-resonance irradiation was performed at 0.75 ppm and off-resonance at 25 ppm where no protein resonances were present. The difference data were obtained by subtraction of the on-resonance fid from the off-resonance. To determine the relative saturation transfer effect integration of the relative peak areas in the 10 sec delay difference spectrum were compared to the peak areas in the 1D-spectrum of **3** or **5**. The individual protons of each inhibitor were referenced to the strongest signal in each difference spectrum, which is assigned as 100%.²⁶ The differential STD effects of the remaining protons within each inhibitor thus yield information on their proximity to the protein.

Sequential resonance assignment: All NMR experiments were performed at 25°C on either a Bruker DRX 600 spectrometer, Varian INOVA 800 indirect-detect triple resonance cryoprobe or a Varian INOVA 500 spectrometer (operating at 499.96 MHz ¹H frequency), equipped with a Varian 5 mm ¹H/¹³C/¹⁵N triple-resonance with Z-axis PFG probe. NMR spectra were processed using the program NMRPipe²⁹ and analyzed with the program Sparky (Goddard and Kneller, 2004). Spectra collected on the Varian INOVA 800: 2D [¹⁵N-¹H] HSQC; 3D spectra: HNCO; HNCA; HNCOCa; CBCA(CO)NH; as well as 3D HNCACB collected on a Bruker 600, were used for sequential assignments of HemO, free and in the presence of **3** or **5**; 3D ¹⁵N-resolved HSQC-NOESY spectra were used to resolve assignment ambiguities and fill in gaps in the sequence.

Chemical shift perturbation—Chemical shift perturbations ¹⁵N-¹H TROSY-selected HSQC experiments were performed on a Varian INOVA 500 at 25°C, using 512 t1 × 4096 (t2) complex points with a width of 1800 and 9000 Hz, respectively. Five samples at 1.2 mM HemO without inhibitor or in the presence of 4.2, 2.1, 1.05, or 0.7 mM were used to collect data. HemO inhibitors **3** and **5** (~4.2 mM) were prepared by addition of 1 mg of inhibitor to 700 μL of HemO 1.2 mM (50 mM phosphate, 100 mM KCl in 95% H₂O : 5% D₂O); after 2 hours at room temperature with occasional vortexing the sample was centrifuged at 5000 x g for 2 minutes to pellet any remaining un-dissolved inhibitor. 600 μL of the soluble inhibitor was transferred to an NMR tube containing 3 μL of TSP (50mM stock in D₂O at a final concentration of 2.2mM total proton). The concentration of inhibitor in the HemO sample is assumed to be the same as that in a mock sample prepared similarly but in the absence of HemO. From this the peak areas of each inhibitor were calculated to be 4.2 mM, relative to the peak area of TSP (2.2mM). The proton and nitrogen resonance variations were followed at 25 °C by (4.2 mM). Combined chemical shift differences between the apo state and each of the titration points were calculated using the following equation (2):

$$\Delta\text{ppm}=\sqrt{(\Delta\delta\text{HN})^2+(\Delta\delta\text{N}\cdot0.2)^2} \quad (2)$$

The nitrogen shift changes are multiplied by a factor of 0.2 to compensate for the difference in chemical shift range between proton and nitrogen. Structural representations and mapping of binding site residues on HemO were generated using PyMOL.³⁰

In silico molecular dynamic simulation and docking studies—Multiple conformations of the heme binding site suitable for ligand docking were obtained from a molecular dynamics (MD) simulation of the apo form of the protein (i.e., without the heme). Preparation of the protein for the simulation involved several steps: (1) obtaining the crystal structure, (2) removal of the heme; (3) solvation of the apo form of the protein; (4)

minimization using the program CHARMM³¹ and (5) equilibration and production simulations using NAMD.³² All computational work used the latest additive CHARMM36 protein force field³³ and the TIP3P water model.³⁴ The initial 3D structure was retrieved from the Protein Data Bank (PDB) (PDB identifier 1SK7) and the heme moiety was removed yielding the apo form of the protein and the structure was solvated using CHARMM-GUI.³⁵ The resulting box of water is at least 10 Å larger than the protein in all directions. All water or sodium ions with non-hydrogen atoms within 2.8 Å of the protein non-hydrogen atoms were subsequently deleted. In the next step CHARMM was used to do the initial energy minimization of the solvent molecules in which 100 steepest descent (SD) steps followed by 200 ABNR steps were performed. The system was then equilibrated in the NpT ensemble ($T = 300$ K, $p = 1$ atm) for 500 ps and a timestep of 2.0 fs followed by a production MD simulation for 80 ns in the NpT ensemble. Periodic boundary conditions were used. Long-range electrostatic interactions were treated using the particle mesh Ewald method with a grid density of approximately $1/\text{\AA}^3$ and a real space cutoff of 12 Å, which was also used for the Lennard-Jones interactions following smoothing over 10 to 12 Å using force switching. SHAKE was used to constrain the covalent bonds involving hydrogen atoms.

Four conformations were selected from the MD simulations and subjected to docking of **3** and **5**. Selection of desired conformations was based on the most “accessible” or “open” state of the heme binding pocket. Suitable conformations were selected by monitoring the minimum distance between then non-hydrogen atoms of the heme axial ligand His26 and the proximal Gly121. Desired conformations correspond to the largest distance between the residues thus allowing the ligand to bind deep in the pocket (Fig S2 of the Supporting Information). Docking against the selected conformations of the apo protein was performed using EAdock³⁶ and SwissDock³⁷ to predict binding modes of **3** and **5**. The docking algorithm relies on the CHARMM22 force field^{38,39} and has been shown to predict crystallographic conformations of bound ligands.⁴⁰ Ligand force field parameters were derived with ParamChem,^{41,42} using the CHARMM General Force Field.⁴³ Binding modes were analyzed using UCSF chimera⁴⁴ and visualized with VMD.⁴⁵

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

HemO	heme oxygenase
Phu	<i>Pseudomonas</i> heme utilization
MIC	minimum inhibitory concentration
cfu	colony forming units
LB	Luria-Bertani
PIA	<i>Pseudomonas</i> isolation agar
SDS-PAGE	sodium dodecyl sulfate-polyacrylamide gel electrophoresis

STD-NMR	saturation transfer difference-nuclear magnetic resonance
HSQC	heteronuclear single quantum correlation
NOESY	nuclear Overhauser effect spectroscopy
TROSY	transverse relaxation optimized spectroscopy
DSS	3(trimethylsilyl) propane-1,1,2,2,3,3- <i>d</i> ₆ -sulfonic acid, sodium salt
TSP	trimethylsilylpropionic acid
CADD	computer aided drug design
MD	molecular dynamics

References

1. Costerton JW. Cystic fibrosis pathogenesis and the role of biofilms in persistent infection. *Trends Microbiol.* 2001; 9:50–52. [PubMed: 11173226]
2. Kerr KG, Snelling AM. *Pseudomonas aeruginosa*: a formidable and ever-present adversary. *J. Hosp. Infect.* 2009; 73:338–344. [PubMed: 19699552]
3. Giske CG, Monnet DL, Cars O, Carmeli Y. Clinical and economic impact of common multidrug-resistant gram-negative bacilli. *Antimicrob. Agents Chemother.* 2008; 52:813–821. [PubMed: 18070961]
4. Rice LB. The clinical consequences of antimicrobial resistance. *Curr. Opin. Microbiol.* 2009; 12:476–481. [PubMed: 19716760]
5. Siegel RE. Emerging gram-negative antibiotic resistance: daunting challenges, declining sensitivities, and dire consequences. *Respir. Care.* 2008; 53:471–479. [PubMed: 18364060]
6. Wilks, A.; Barker, KD. *Handbook of Porphyrin Science*. 1st ed. Kadish, KM.; Smith, KM.; Guillard, R., editors. Vol. 15. World Scientific; Singapore: 2011. p. 357-398.
7. Wilks A, Burkhard KA. Heme and virulence: how bacterial pathogens regulate, transport and utilize heme. *Nat. Prod. Rep.* 2007; 24:511–522. [PubMed: 17534527]
8. Skaar EP, Humayun M, Bae T, DeBord KL, Schneewind O. Iron-source preference of *Staphylococcus aureus* infections. *Science.* 2004; 305:1626–1628. [PubMed: 15361626]
9. Brickman TJ, Vanderpool CK, Armstrong SK. Heme transport contributes to in vivo fitness of *Bordetella pertussis* during primary infection in mice. *Infect. Immun.* 2006; 74:1741–1744. [PubMed: 16495546]
10. Ochsner UA, Johnson Z, Vasil ML. Genetics and regulation of two distinct haem-uptake systems, phu and has, in *Pseudomonas aeruginosa*. *Microbiology.* 2000; 146(Pt 1):185–198. [PubMed: 10658665]
11. Krieg S, Huche F, Diederichs K, Izadi-Pruneyre N, Lecroisey A, Wandersman C, Delepelaire P, Welte W. Heme uptake across the outer membrane as revealed by crystal structures of the receptor-hemophore complex. *Proc. Natl. Acad. Sci. U S A.* 2009; 106:1045–1050. [PubMed: 19144921]
12. Letoffe S, Delepelaire P, Wandersman C. Free and hemophore-bound heme acquisitions through the outer membrane receptor HasR have different requirements for the TonB-ExbB-ExbD complex. *J. Bacteriol.* 2004; 186:4067–4074. [PubMed: 15205408]
13. Letoffe S, Deniau C, Wolff N, Dassa E, Delepelaire P, Lecroisey A, Wandersman C. Haemophore-mediated bacterial haem transport: evidence for a common or overlapping site for haem-free and haem-loaded haemophore on its specific outer membrane receptor. *Mol. Microbiol.* 2001; 41:439–450. [PubMed: 11489129]
14. Ratliff M, Zhu W, Deshmukh R, Wilks A, Stojiljkovic I. Homologues of Neisserial Heme Oxygenase in Gram-Negative Bacteria: Degradation of Heme by the Product of the *pigA* Gene of *Pseudomonas aeruginosa*. *J. Bacteriol.* 2001; 183:6394–6403. [PubMed: 11591684]

15. Furci LM, Lopes P, Eakanunkul S, Zhong S, MacKerell AD Jr, Wilks A. Inhibition of the bacterial heme oxygenases from *Pseudomonas aeruginosa* and *Neisseria meningitidis*: novel antimicrobial targets. *J. Med. Chem.* 2007; 50:3804–3813. [PubMed: 17629261]
16. Bjorn MJ, Vasil ML, Sadoff JC, Iglewski BH. Incidence of exotoxin production by *Pseudomonas* species. *Infect. Immun.* 1977; 16:362–366. [PubMed: 68931]
17. Ohman DE, Chakrabarty AM. Genetic mapping of chromosomal determinants for the production of the exopolysaccharide alginate in a *Pseudomonas aeruginosa* cystic fibrosis isolate. *Infect. Immun.* 1981; 33:142–148. [PubMed: 6790439]
18. Dalhoff A, Ambrose PG, Mouton JW. A long journey from minimum inhibitory concentration testing to clinically predictive breakpoints: deterministic and probabilistic approaches in deriving breakpoints. *Infection.* 2009; 37:296–305. [PubMed: 19629383]
19. Maglio D, Nicolau DP. The integration of pharmacokinetics and pathogen susceptibility data in the design of rational dosing regimens. *Methods Find. Exp. Clin. Pharmacol.* 2004; 26:781–788.
20. Moy TI, Ball AR, Anklesaria Z, Casadei G, Lewis K, Ausubel FM. Identification of novel antimicrobials using a live-animal infection model. *Proc. Natl. Acad. Sci. U S A.* 2006; 103:10414–10419. [PubMed: 16801562]
21. Roman G, Riley JG, Vlahakis JZ, Kinobe RT, Brien JF, Nakatsu K, Szarek WA. Heme oxygenase inhibition by 2-oxy-substituted 1-(1H-imidazol-1-yl)-4-phenylbutanes: effect of halogen substitution in the phenyl ring. *Bioorg. Med. Chem.* 2007; 15:3225–3234. [PubMed: 17339115]
22. Vlahakis JZ, Kinobe RT, Bowers RJ, Brien JF, Nakatsu K, Szarek WA. Imidazole-dioxolane compounds as isozyme-selective heme oxygenase inhibitors. *J. Med. Chem.* 2006; 49:4437–4441. [PubMed: 16821802]
23. Lad L, Schuller DJ, Shimizu H, Friedman J, Li H, Ortiz de Montellano PR, Poulos TL. Comparison of the heme-free and -bound crystal structures of human heme oxygenase-1. *J. Biol. Chem.* 2003; 278:7834–7843. [PubMed: 12500973]
24. La Mar GN, Asokan A, Espiritu B, Yeh DC, Auclair K, Ortiz De Montellano PR. Solution 1H NMR of the active site of substrate-bound, cyanide-inhibited human heme oxygenase. Comparison to the crystal structure of the water-ligated form. *J. Biol. Chem.* 2001; 276:15676–15687.
25. Friedman J, Lad L, Li H, Wilks A, Poulos TL. Structural basis for novel delta-regioselective heme oxygenation in the opportunistic pathogen *Pseudomonas aeruginosa*. *Biochemistry.* 2004; 43:5239–5245. [PubMed: 15122889]
26. Beanan MJ, Strome S. Characterization of a germ-line proliferation mutation in *C. elegans*. *Development.* 1992; 116:755–766. [PubMed: 1289064]
27. Kluger R, Hunt J. Aminolysis of Maleic Anhydride. Kinetics and Mechanism of Amide Formation. *J. Am. Chem. Soc.* 1984; 106:5667–5670.
28. Mayer M, Meyer B. Group epitope mapping by saturation transfer difference NMR to identify segments of a ligand in direct contact with a protein receptor. *J. Am. Chem. Soc.* 2001; 123:6108–6117. [PubMed: 11414845]
29. Delaglio F, Grzesiek S, Vuister GW, Zhu G, Pfeifer J, Bax A. NMRPipe: a multidimensional spectral processing system based on UNIX pipes. *J. Biomol. NMR.* 1995; 6:277–293. [PubMed: 8520220]
30. The PyMOL Molecular Graphics System. V. r. p., Schrödinger;
31. Brooks BR, Brooks CL 3rd, Mackerell AD Jr, Nilsson L, Petrella RJ, Roux B, Won Y, Archontis G, Bartels C, Boresch S, Caflisch A, Caves L, Cui Q, Dinner AR, Feig M, Fischer S, Gao J, Hodoscek M, Im W, Kuczera K, Lazaridis T, Ma J, Ovchinnikov V, Paci E, Pastor RW, Post CB, Pu JZ, Schaefer M, Tidor B, Venable RM, Woodcock HL, Wu X, Yang W, York DM, Karplus M. CHARMM: the biomolecular simulation program. *J. Comp. Chem.* 2009; 30:1545–1614. [PubMed: 19444816]
32. Phillips JC, Braun R, Wang W, Gumbart J, Tajkhorshid E, Villa E, Chipot C, Skeel RD, Kale L, Schulten K. Scalable molecular dynamics with NAMD. *J. Comp. Chem.* 2005; 26:1781–1802. [PubMed: 16222654]
33. Best R, Zhu X, Shim J, Lopes P, Mittal J, Feig M, Mackerell AD Jr. Optimization of the Additive CHARMM All-Atom Protein Force Field Targeting Improved Sampling of the Backbone and Side-Chain Dihedral Angles. *J. Chem. Theory Comput.* 2012; 8:3257–3273.

34. Jorgensen W, Chandrasekhar J, Madura J, Impey R, Klein M. Comparison of Simple Potential Functions for Simulating Liquid Water. *J. Chem. Phys.* 1983; 79:926–935.
35. Jo S, Kim T, Iyer VG, Im W. CHARMM-GUI: a web-based graphical user interface for CHARMM. *J. Comp. Chem.* 2008; 29:1859–1865. [PubMed: 18351591]
36. Grosdidier A, Zoete V, Michielin O. EADock: docking of small molecules into protein active sites with a multiobjective evolutionary optimization. *Proteins.* 2007; 67:1010–1025. [PubMed: 17380512]
37. Grosdidier A, Zoete V, Michielin O. SwissDock, a protein-small molecule docking web service based on EADock DSS. *Nucleic Acids Res.* 2011; 39:W270–277. [PubMed: 21624888]
38. Brooks BR, Bruccoleri RE, Olafson BD, States DJ, Swaminathan S, Karplus M. CHARMM: A Program for Macromolecular Energy, Minimization, and Dynamics Calculations. *J. Comp. Chem.* 1983; 4:187–217.
39. MacKerell AD Jr, Bashford D, Bellott M, Dunbrack RL Jr, Evanseck J, Field MJ, Fischer S, Gao J, Guo H, Ha S, Joseph D, Kuchnir L, Kuczera K, Lau FTK, Mattos C, Michnick S, Ngo T, Nguyen DT, Prodhom B, Reiher I, W. E, Roux B, Schlenkrich M, Smith J, Stote R, Straub J, Watanabe M, Wiorkiewicz-Kuczera J, Yin D, Karplus M. All-atom empirical potential for molecular modeling and dynamics studies of proteins. *J. Phys. Chem. B.* 1998; 102:3586–3616.
40. Zoete V, Grosdidier A, Cuendet M, Michielin O. JUSe of the FACTS solvation model for protein-ligand docking calculations. Application to EADock. *J. Mol. Recognit.* 2010; 23:457–461. [PubMed: 20101644]
41. Vanommeslaeghe K, Mackerell AD Jr. Automation of the CHARMM General Force Field (CGenFF) I: Bond Perception and Atom Typing. *J. Chem. Inf. Model.* 2012; 52:3144–3154. [PubMed: 23146088]
42. Vanommeslaeghe K, Raman EP, Mackerell AD Jr. Automation of the CHARMM General Force Field (CGenFF) II: Assignment of Bonded Parameters and Partial Atomic Charges. *J. Chem. Inf. Model.* 2012; 52:3155–3168. [PubMed: 23145473]
43. Vanommeslaeghe K, Hatcher E, Acharya C, Kundu S, Zhong S, Shim J, Darian E, Guvench O, Lopes P, Vorobyov I, Mackerell AD Jr. CHARMM general force field: A force field for drug-like molecules compatible with the CHARMM all-atom additive biological force fields. *J. Comp. Chem.* 2010; 31:671–690. [PubMed: 19575467]
44. Pettersen EF, Goddard TD, Huang CC, Couch GS, Greenblatt DM, Meng EC, Ferrin TE. UCSF Chimera—a visualization system for exploratory research and analysis. *J. Comp. Chem.* 2004; 25:1605–1612. [PubMed: 15264254]
45. Humphrey W, Dalke A, Schulten K. VMD: visual molecular dynamics. *J. Mol. Graphics Modell.* 1996; 14:33–38.

Figure 1. Fluorescence emission spectra of apo-HemO on incremental addition of 3 (A) and 4 (B) To 1.0 μM apo-HemO in 20 mM Tris-HCl (pH 7.4) inhibitor 3 or 4 was added in increments from 0.05 to 200 μM . The binding constant (K_d) was fit to a one-site binding model (inset) according to the decrease in Trp fluorescence at 332 nm as a function of inhibitor concentration.

Figure 2. STD-NMR spectra for 3 and 5

A. STD spectrum of inhibitor **3** with apo-HemO. Signals are observed for all assignable protons. **B.** Reference spectrum and assignments for inhibitor **3**. **C.** Binding epitope for inhibitor **3**. **D.** STD spectrum of inhibitor **5** with apo-HemO. **E.** Reference spectrum and assignments for inhibitor **5**. **F.** Binding epitope for inhibitor **5**. Protons detected in the difference spectra shown in bold italics. STD-NMR spectra for inhibitor **4** was identical to that of **3** and is therefore not shown.

Figure 3. Chemical shift perturbations of apo-HemO on interaction with 3

A. Plot of the weighted averages of the ^1H and ^{15}N chemical shifts. Bars shown in orange are those residues assigned only in the inhibitor bound HemO and they have been arbitrarily set at the maximum chemical shift. The absence of bars in the plot indicates residues assigned in neither the apo- or inhibitor bound HemO. Sequences within the heme binding pocket and distal loops are highlighted and residues assigned only in the inhibitor bound HemO are shown in bold. **B.** Mapping of the perturbed residues onto the crystal structure of holo-HemO (PDB entry 1SK7). The heme is not shown however His-26 and a distal Gly-121 are highlighted in cyan as a reference for the heme binding cavity. Residues highlighted in orange and yellow are those residues that are assigned only in the inhibitor bound enzyme (orange) or residues with the greatest magnitude in chemical shifts (yellow). Residues of importance whose resonances are no longer visible in the inhibitor bound HemO are shown in blue. **C.** Same as in B rotated by 90 °C along the y-axis.

Figure 4. Chemical shift perturbations of apo-HemO on interaction with 5

A. Plot of the weighted averages of the ^1H and ^{15}N chemical shifts. Bars shown in orange are those residues from the distal and C-terminal helix that form the distal pocket. The absence of bars in the plot indicates residues assigned in neither the apo- or inhibitor bound HemO. Sequences of the distal, C-terminal, and flexible surface loop are highlighted. Surface exposed residues are shown in bold. **B.** Mapping of the perturbed residues onto the crystal structure of holo-HemO (PDB entry 1SK7). The heme is not shown however His-26 is highlighted in cyan as a reference for the heme binding cavity. Residues highlighted in orange and yellow are the surface exposed residues. Residues showing the greatest magnitude in chemical shifts are shown in orange with the second level shifts in yellow. **C.** Same as in B rotated by 180 °C along the x-axis.

Figure 5. NMR titration analysis of HemO with **3 and **5****

A. A section of five overlaid ^1H - ^{15}N TROSY-HSQC spectra of ^{15}N -labelled apo-HemO (1.2 mM) during titration with inhibitor **3**. *Green*, 0 mM inhibitor **3**; *Red*, 0.7 mM inhibitor **3**; *Yellow*, 1.05 mM inhibitor **3**; *Cyan*, 2.1 mM inhibitor **3**; *Blue*, 4.2 mM inhibitor **3**. **B.** Chemical shift for select residues on the proximal helix and distal loops of apo-HemO as a function of increasing concentration of **3**. **C.** Chemical shift for select distal helix and surface residues of apo-HemO as a function of increasing concentration of **5**.

Figure 6. Images of 3 bound to HemO in the four conformations obtained from the MD simulation

Surface representation of heme oxygenase viewing directly into the heme binding pocket with the three top docked poses shown for each conformation. Protein residues Lys-34 and Phe-39 are shown in green and His-26 and Gly-121 as grey space filling representations. The docked Inhibitor **3** is shown in licorice format with each pose being assigned a separate color as indicated. Only two poses are observed in the vicinity of the binding pocket for the 52.0 ns conformation.

Figure 7. Images of 5 bound to HemO in the four conformations obtained from the MD simulation

Surface representation of heme oxygenase viewing directly into the heme binding pocket with the three top docked poses shown for each conformation. Protein residues Lys-34 and Phe-39 are shown in green and His-26, Phe-55 and Gly-121 as grey space filling representations. The docked Inhibitor 5 is shown in licorice format with each pose being assigned a separate color as indicated. Only two poses are observed in the vicinity of the binding pocket for the 11.38 and 75.92 ns conformations.

Figure 8. Curing of a *C. elegans* *P. aeruginosa* infection with 5

A. Survival curves of synchronized *glp-4(bn2)* worms fed on *P. aeruginosa* for 18 hours and subsequently transferred to *E. coli* OP50 plates containing 0 $\mu\text{g/ml}$ inhibitor 5 (◆) or 250 $\mu\text{g/ml}$ inhibitor 3 (●), uninfected worms on 250 $\mu\text{g/ml}$ inhibitor 3 (■) ($P < 0.001$). **B.** Bacterial load in the worm intestinal tract. Worms treated with (hashed bars) or without inhibitor (solid bars) were washed and the cfu calculated by serial dilution. Error bars represent the standard deviation.

Scheme 1.0.
Structures and synthesis of apo-HemO inhibitors.

Table 1

Binding affinities and MIC values for apo-HemO inhibitors.

Inhibitor	K_D (μ M)	MIC ₅₀ (μ g/ml)		
		PAO1	WR5	FRD1
3	18.4 $\pm$ 2.4	1.2 $\times$ 10 ³	1.1 $\times$ 10 ³	950
4
5	29.3 $\pm$ 4.4	1.0 $\times$ 10 ³	800	750
	31.2 $\pm$ 2.9	250	210	190

Table 2

Distance between the center of mass of the apo-HemO phenyl ring of Phe-55 and NZ of Lys-34 and the center of mass of the terminal phenyl ring (Phe) and carbon of the terminal carboxylate (COO⁻) of inhibitor **3**.

Inhibitor 3 pose number	apo-HemO conformation							
	11.38 ns		14.74 ns		52.00 ns		75.92 ns	
	Phe55- Phe (Å)	Lys34- COO ⁻ (Å)	Phe55- Phe (Å)	Lys34- COO ⁻ (Å)	Phe55- Phe (Å)	Lys34- COO ⁻ (Å)	Phe55- Phe (Å)	Lys34- COO ⁻ (Å)
1	6.96	4.63	12.29	11.96	9.17	2.17	8.97	11.74
2	6.14	7.96	6.96	5.85	9.04	2.19	9.02	11.05
3	11.08	5.08	7.76	4.24	*na	*na	8.66	3.89

Table 3

Distance between the center of mass of the apo-HemO phenyl ring of Phe-55 and the center of mass of the terminal dimethyl amino (NMe₂) and carbon of the terminal hydrazide of inhibitor **5**.

Inhibitor pose number	apo-HemO conformation							
	11.38 ns		14.74 ns		52.00 ns		75.92 ns	
	Phe55-N(Me) ₂ (Å)	Phe55-C(=NH)(NH ₂)(Å)	Phe55-N(Me) ₂ (Å)	Phe55-C(=NH)(NH ₂)(Å)	Phe55-N(Me) ₂ (Å)	Phe55-C(=NH)(NH ₂)(Å)	Phe55-N(Me) ₂ (Å)	Phe55-C(=NH)(NH ₂)(Å)
1	10.46	13.40	8.98	8.98	*na	*na	*na	*na
2	6.56	13.69	10.30	7.86	9.68	18.11	*na	*na
3	*na	*na	6.96	11.58	9.64	10.57	17.57	11.21