Discovery of stimulator binding to a conserved pocket in the heme domain of soluble guanylyl cyclase

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Running Title: Stimulator binding to the sGC \(\beta\)I H-NOX domain

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\textbf{ABSTRACT}

Soluble guanylyl cyclase (sGC) is the receptor for nitric oxide and a highly sought-after therapeutic target for the management of cardiovascular diseases. New compounds that stimulate sGC show clinical promise, but where these stimulator compounds bind and how they function remains unknown. Here, using a photoaffinity derivatized derivative of a novel stimulator compound, IWP-051, and MS analysis, we localized drug binding to the \(\beta\)I heme domain of sGC proteins from the hawkmoth \textit{Manduca sexta} and from human. Covalent attachments to the stimulator were also identified in bacterial homologs of the sGC heme domain, referred to as H-NOX domains, including those from \textit{Nostoc sp. 7120}, \textit{Shewanella oneidensis}, \textit{Shewanella woodyi}, and \textit{Clostridium botulinum}, indicating the binding site is highly conserved. The identification of photoaffinity-labeled peptides was aided by a signature MS fragmentation pattern of general applicability for unequivocal identification of covalently attached compounds. Using NMR, we also examined stimulator binding to sGC from \textit{M. sexta} and bacterial H-NOX homologs. These data indicated that stimulators bind to a conserved cleft between two subdomains in the sGC heme domain. L23W/T48W substitutions within the binding pocket resulted in a 9-fold decrease in drug response, suggesting the bulkier tryptophan residues directly block stimulator binding. The localization of stimulator binding to the sGC heme domain reported here resolves the longstanding question of where stimulators bind and provides a path forward for drug discovery.

Nitric oxide (NO) signaling is compromised in numerous forms of vascular pathology (1,2) and the components of NO signaling pathways are highly sought after therapeutic targets (3). Central to NO signaling is soluble guanylyl cyclase (sGC), the NO receptor, which regulates vascular tone, platelet activation, wound healing and other factors of importance to cardiovascular health (4,5). sGC is an ~150 kDa heterodimeric NO sensor composed of \(\alpha\) and \(\beta\) subunits, with an \(\alpha1/\beta1\) isoform predominating in vascular tissue (also referred to as guanylyl cyclase-1, GC-1), and an \(\alpha2/\beta1\) isoform predominating in nerve cells (called GC-2), where it is important for memory formation. The sGC \(\beta1\) subunit contains an N-terminal Heme-Nitric Oxide / Oxygen binding (H-NOX) domain, a Per-ARNT-Sim (PAS) domain, a coiled-coil domain and a catalytic cyclase homology domain (Fig. 1A). The sGC \(\alpha1\) subunit was likely formed through gene duplication (5) and retains a similar domain arrangement to the \(\beta1\) subunit except that the H-NOX domain has lost the ability to bind heme and is best referred to as a pseudo H-NOX domain.

sGC binds NO on a ferrous heme in the \(\beta1\) H-NOX domain, leading to allosteric stimulation of cyclase activity, production of cyclic...
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guanosine-3’,5’-monophosphate (cGMP) from guanosine-triphosphate (GTP) and a downstream signaling cascade. A single active site is formed at the interface of the α and β cyclase homology domains, with each subunit contributing amino acids necessary for catalysis. sGC is therefore an obligate heterodimer.

sGC is targeted pharmacologically to treat numerous vascular disorders, including acute coronary syndromes, congestive heart failure, and arterial hypertension, through the use of NO donors and organic nitrates (6). While these compounds exhibit potent vasodilatory and anti-ischemic effects, tolerance readily develops and cellular damage by excess NO can occur (6). More recently, compounds that increase cGMP production without altering cellular levels of NO have been sought.

sGC stimulators were the first compounds to overcome the limitations of NO donors and organic nitrates by enhancing cyclase activity both independently and synergistically with NO (3,7-10). Optimization of initial stimulator compounds led to the development of BAY 41-2272, which is widely used for investigating stimulator mechanism, and BAY 63-2521 (riociguat), which is clinically approved to treat pulmonary arterial hypertension (PAH) and chronic thromboembolic pulmonary hypertension (CTEPH) (marketed as Adempas) (3). Additional sGC stimulators have been developed, including IWP-051, a novel compound representing a new class of stimulators with improved solubility over traditional stimulators and favorable pharmacodynamics properties (11).

Despite much success and compounds in the clinic, where sGC stimulators bind and how they function remains unknown. Here, we identify binding of stimulator compounds to the sGC heme domain and bacterial H-NOX homologs using NMR approaches and a unique photoactive labeling stimulator with a signature cleavage pattern that allows for unambiguous LC-MS/MS peptide assignment.

RESULTS

Development of a photolyzable stimulator,
IWP-854—To localize stimulator binding to sGC, we synthesized a photolabile compound called IWP-854. The IWP-854 core motif is based on IWP-051, which replaces the 7-azaindazole core of BAY 41-2272 with pyrazole-isoxazole 5-membered rings capable of free rotation (Fig. 1B) (11). Previous studies indicate altering the benzyl ring abolishes binding while modifications to the pyrimidine ring are widely tolerated (11,12). With this in mind, we modified the pyrimidine ring to have a biotin affinity tag coupled to a PEG linker and a photoactive diazirine capable of covalently attaching to all 20 amino acid side chains and peptide backbone (13-15) (Fig. 1B). Our synthetic scheme is described in the SI Appendix.

IWP-854 retains stimulator activity—We examined stimulation of recombinant human (Hs) sGC and found IWP-854 to stimulate to a similar extent as BAY 41-2272 (Fig. 2A). Both basal and NO stimulated activities were enhanced, as previously described for sGC stimulators (7,8,16). IWP-854 increased activity by 12-fold over basal levels in the absence of added NO, and 84-fold over basal upon addition of NO.

We previously developed truncated versions of sGC from Manduca sexta for analyses of compound-enhanced CO binding (17-20), which we refer to as Ms sGC-NT (Fig. 1A). Ms sGC-NT constructs are fully heme-loaded and stable in the ferrous (functional) state, as indicated by Soret band absorption (supplemental Fig. S1). One hallmark of stimulator compounds is their ability to enhance CO and NO binding to the heme domain (reviewed in (5)). CO binding to Ms sGC-NT23 in the absence of stimulator compound displays $K_d^{CO} = 710$ nM (Fig. 2B). Addition of BAY 41-2272 enhances CO affinity 14-fold ($K_d^{CO} = 52$ nM) while addition of IWP-854 enhances CO affinity 34-fold ($K_d^{CO} = 21$ nM). Thus, IWP-854 stimulates as well or somewhat better than the best previously described compounds.

IWP-854 and BAY 41-2272 share a common binding site—Labeling with IWP-854 was visualized by probing the biotin affinity tag through western blot (Fig. 2C–E). A time course revealed that labeling to Ms sGC-NT23 is observed after 5 minutes of UV illumination and continues to increase for 15-20 minutes (supplemental Fig. S2). Heme was retained after 15 minutes of UV irradiation, as indicated by a minimal decrease in Soret band absorption and a slight shift in Soret maxima characteristic of stimulator binding (20) (supplemental Fig. S3). Longer exposures to UV light led to substantial
heme loss, however, and were avoided for this reason. Using this strategy, we found that IWP-854 exclusively labels the β1 subunit of Ms sGC-NT23 (Fig. 2C).

To assess compound specificity, IWP-854 labeling was monitored in the presence of increasing concentrations of BAY 41-2272 (Fig. 2C). Incubation with BAY 41-2272 attenuated IWP-854 labeling of Ms sGC-NT23 in a concentration-dependent manner, indicating the two compounds were competing for a single binding pocket. Similar results were observed for Ms sGC-NT13 (supplemental Fig. S4A), which includes the α1 pseudo H-NOX domain, and full length Hs sGC (Fig. 2D). Faint labeling was observed on the α1 subunit of Hs sGC; however, this labeling was not competed away with excess stimulator and is likely due to non-specific labeling. IWP-854 also labeled the isolated β1 subunit of Ms sGC-NT21 (β1 residues 1-380, supplemental Fig. S4B). Curiously, BAY 41-2272 failed to diminish IWP-854 labeling of Ms sGC-NT21 β1; however, IWP-854 labeled the same residues in Ms sGC-NT21 β1 as in other Ms sGC-NT constructs (described below).

Yoo and colleagues previously reported that BAY 41-2272 not only alters the release kinetics of CO from sGC, but also from a homologous bacterial H-NOX domain from Clostridium botulinum (21), commonly referred to as Cb SONO (22). We therefore characterized stimulator binding to Cb SONO, as well as three previously described H-NOX proteins from Nostoc sp. 7120 (Ns H-NOX), Shewanella oneidensis (So H-NOX), and Shewanella woodyi (Sw H-NOX) (23). IWP-854 labeled all four bacterial H-NOX proteins (Fig. 2E, supplemental Fig. S4C), suggesting stimulator binding is conserved among β1 H-NOX domains. Photoaffinity labeling of bacterial H-NOX proteins required 10-fold more IWP-854 than sGC constructs, indicating decreased affinity for compound binding. Labeling was reduced but not eliminated by excess BAY 41-2272, which is likely explained by the inability to reach sufficiently high BAY 41-2272 concentrations due to poor compound solubility, and by the increased nonspecific labeling that occurs at higher IWP-854 concentrations. Interestingly, BAY 41-2272 (10 μM) did not enhance CO binding to any of the four bacterial H-NOX proteins (supplemental Table S2). This may be due to the weaker compound binding but is also consistent with previous results with sGC, where stimulator enhanced CO binding to the heterodimeric protein, but not to the isolated H-NOX domain (20).

Identifying labeled residues in sGC and bacterial H-NOX proteins—Residues labeled by IWP-854 were identified by liquid chromatography tandem mass spectrometry (LC-MS/MS) using an LTQ Velos Orbitrap mass spectrometer (Thermo). Initial examination of IWP-854 alone (molecular mass 1,450.743 Da) revealed a distinct and highly reproducible fragmentation pattern (supplemental Fig. S5). Key features include a singly-charged peak at m/z 270.127 and a peak one charge state less than the precursor representing the mass of the parent ion minus 270.127 Da. MS² analysis of m/z 270.127 identified the fragment as the end of the biotin-containing PEG linker (C₁₂H₂₅O₇N₅S, Fig. 1B and supplemental Fig. S5). This signature cleavage pattern was consistently observed in labeled peptides (Fig. 3A), providing a robust strategy for definitively identifying peptides modified by IWP-854. A possible mechanism for the characteristic fragmentation pattern is for the linker amide near the cleavage site to yield a localized mobile proton that assists in the cleavage event (24), thus reproducibly generating the m/z 270.127 fragment. More broadly, judicious placement of an amide bond in a PEG linker may provide a unique cleavage pattern of general applicability.

The availability of multiple Ms sGC-NT constructs in high purity and abundance allowed for numerous experiments to be undertaken under varying conditions. Hs sGC and four bacterial H-NOX proteins were also examined using similar conditions to those initially developed with Ms sGC-NT. Results from a total of 43 experiments are reported in Table 1 and supplemental Table S3. Representative sequence coverage for each protein is depicted in supplemental Fig. S6.

Identification of peptides was to high mass accuracy in all cases; however, certain peptides were detected more often than others (Table 1). Most labeled residues identified in Ms sGC-NT and Hs sGC are expected to originate from the stimulator-binding pocket, as evidenced by diminished labeling in the presence of excess BAY 41-2272 (Fig. 2C). In general, the diversity
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in labeling of sGC likely results from dynamics in both compound and protein. For IWP-854, the diazirine is at the fourth carbon of a 5-atom flexible linker attached to a pyrimidine ring capable of free rotation (Fig. 1B). The pyrimidine ring was previously shown to be tolerant to some modification (11), suggesting it may exhibit greater conformational dynamics than the rest of the compound, even in the bound state.

Labeling of \( Ms \) sGC-NT23 remained the same in the presence or absence of NO or CO, consistent with a stimulator binding site that does not greatly change upon heme ligation. Likewise, labeling did not appreciably differ in the presence (\( Ms \) sGC-NT13) or absence (\( Ms \) sGC-NT23) of the \( \alpha1 \) pseudo H-NOX domain, indicating this domain does not harbor the stimulator binding site. IWP-854 labeling of \( Ms \) sGC-\( \beta1 \) is similar to the other \( Ms \) sGC-NT constructs despite lacking the \( \alpha1 \) chain and displaying poor competition with BAY 41-2272. Labeled peptides identified in the \( Ms \) sGC-NT constructs agreed well with those in full-length \( Hs \) sGC and were found nearly exclusively in the \( \beta1 \) subunit, as expected from the western blot analyses. Likewise, many labeled peptides identified in the bacterial H-NOX proteins overlapped with those from sGC.

The most frequently observed labels cluster together in our previous model for \( Ms \) sGC-NT (Fig. 3B) (19). IWP-854 predominately labeled the sGC \( \beta1 \) chain on H-NOX alpha helix \( \alphaA \) (\( Ms \) residues \( \beta1 \) 6-8), H-NOX helix \( \alphaC \) (\( Hs \) residues \( \beta1 \) 48-51), and the coiled-coil domain (\( Ms \) residues \( \beta1 \) 361-362, 365-366; \( Hs \) residues \( \beta1 \) 370-371, 374-375, 385). Labeling of helix \( \alphaA \) was also seen for the bacterial H-NOX proteins \( Cb \) SONO and \( Sw \) H-NOX, while labeling of helix \( \alphaC \) was seen for \( Ns \) H-NOX, \( So \) H-NOX, and \( Sw \) H-NOX. Helix \( \alphaD \) was not labeled in any sGC constructs but was detected in bacterial H-NOX proteins \( Ns \) H-NOX, \( Sw \) H-NOX, and \( Cb \) SONO. Labeled residues in \( \alphaA \), \( \alphaC \), and \( \alphaD \) localize around the interface of two subdomains in the \( \beta1 \) H-NOX and are predicted to reside near labeled residues in the coiled-coil and the linker connecting the PAS and coiled-coil domains of \( Ms \) sGC-NT (\( Ms \) residues \( \beta1 \) 328-331) (19).

Labeling of residues \( Ms \) sGC-NT \( \beta1 \) 195-198 was seen in 11 of 26 measurements; however, these residues lie in the linker between the H-NOX and PAS domains and in a different region of our model. This discrepancy could be due to limitations in our modeling, a slight degree of nonspecific binding or high dynamics in this loop.

A number of additional labeled peptides were detected on a less frequent basis in these experiments, and are listed in Table 1 and supplemental Table S3. Modifications to the \( \beta1 \) H-NOX/PAS linker were observed in all three \( Ms \) sGC-NT constructs, but not \( Hs \) sGC. Additionally, a variety of labels were detected in individual bacterial H-NOX proteins that do not agree with the most common binding arrangement. These are likely the result of unspecific labeling introduced by increased compound concentrations and/or changes in compound affinity, as evidenced by incomplete elimination of IWP-854 labeling by competition with BAY 41-2272. For this reason, only labeled residues that were identified in multiple bacterial H-NOX proteins were considered to be part of the binding site.

Stimulators have been proposed to bind to a pseudosymmetric site in the cyclase domains similar to forskolin binding to adenylyl cyclase (25,26). \( Ms \) sGC-NT constructs retain stimulator binding and response despite lacking both cyclase domains, suggesting the primary stimulator binding site resides in the N-terminal two-thirds of the protein (18-20). We examined the possibility of a secondary stimulator binding site in the catalytic domains using photoaffinity labeling of full length \( Hs \) sGC. A single label was found in the cyclase domain (\( Hs \) residue \( \alpha1 \) 629, supplemental Table S3), which lies on the surface of the protein near where the coiled-coil attaches. No labeling was found of residues in the cyclase domain active site or pseudosymmetric site, rendering the possibility of a secondary stimulator binding site unlikely. One additional label to the human \( \alpha1 \) chain was observed (\( Hs \) peptide \( \alpha1 \) 45-47, Table S3). The two \( \alpha1 \) chain labels identified by mass spectrometry may represent the nonspecific \( \alpha1 \) labeling observed by western blotting.

Characterization of compound binding by transferred NOESY NMR—IWP-051 binding to \( Ms \) sGC-NT23, \( Cb \) SONO, and \( Sw \) H-NOX was further examined by transferred nuclear Overhauser effect spectroscopy (TrNOESY). TrNOESY provides information on the protein-bound conformation of the compound by
measuring proton-proton NOE cross-relaxation enhancement. Upon binding the protein, the ligand acquires a large molecular correlation time leading to substantial enhancement of NOE cross relaxation between protons. Thus, protein-bound compounds exhibit strong negative NOEs and significantly shorter mixing times, compared to the small positive NOEs and longer mixing times exhibited by free ligand (27).

The NOESY spectrum of IWP-051 (Fig. 4A) in the absence of protein displayed weak positive NOEs at longer mixing times (>1.2 seconds) (Fig. 4B upper panel, supplemental Table S4). Prominent NOEs include those between hydrogens in the benzyl ring (H13, H14, H15 and H16) and the isoxazole ring (H9 and H10). Most NOEs involving the methylene hydrogens (H11) were not observed, consistent with high rotational dynamics in the methylene bridge and benzyl ring. No NOE peaks were observed at shorter mixing times (<1.0 second).

Upon adding Ms sGC-NT23, strong negative NOEs appeared at a short mixing time typical for protein molecules (400 ms). Three new NOEs were observed for protein-bound IWP-051, all involving the methylene bridging hydrogens (H2-H11, H9-H11 and H11-H14; Fig. 4B lower panel). One NOE peak (H10-H20) was lost. As a control, we examined binding by compound PF-04447943, a phosphodiesterase 9A (PDE9A) inhibitor (28) that does not stimulate sGC. No NOE peaks were observed with this compound (supplemental Fig. S7). Together, these data suggest specific binding for IWP-051 to Ms sGC-NT23. Approximate proton-proton distances for IWP-051 derived from TrNOESY are reported in supplemental Table S4 with comparison to the distances from the model structure, which are in good agreement. IWP-051 binding to Cb SONO and Sw H-NOX were also examined, with both proteins generating negative TrNOESY peaks. However, the spectra display weaker intensities than Ms sGC-NT23, likely due to the smaller sizes of the bacterial H-NOX proteins (supplemental Fig. S7).

These data reveal several key factors related to stimulator binding. First, a strong NOE peak between two protons (H2-H10) was observed upon binding (Fig. 4B), suggesting the isoxazole and pyrazole rings are roughly planar and the protons from each ring lie near one another. Since the magnitude of NOE peaks decrease rapidly with distance and are only measurable to ~6 Å, the two rings must be nearly co-planar for a strong TrNOESY peak to appear in IWP-051. Second, there is a weak TrNOESY peak between an isoxazole proton and the methylene protons (H10-H11, Fig. 4B), consistent with the orientation shown in the figure and the previously reported structure-activity relationship (SAR) of IWP-051 (11). Third, the appearance of several TrNOESY peaks for the benzyl ring indicates it occupies a single conformation in the binding pocket. Finally, and most importantly, stimulator binding is conserved from bacterial H-NOX proteins to human sGC.

**Chemical shift perturbation in Sw H-NOX HSQC indicates binding pocket—**Sw H-NOX displays well-dispersed HSQC spectra and 75% of the backbone assignments have been reported (29). We therefore completed the backbone assignment and undertook chemical shift perturbation analyses to uncover which residues respond to binding IWP-051 (Figs. 5A-C, S8 and S9, supplemental Tables S5 and S6). Several residues displayed prominent concentration-dependent shifts in resonance upon titration with IWP-051 (Fig. 5B), but not with PDE9A inhibitor. The largest shifts cluster together near the C-terminal end of helix αA (Sw residues 14-18) and the N-terminal end of helix αD (Sw residues 61-76, Fig. 5C). These regions are in contact in our homology model (Fig. 5A, described below). Interestingly, they also reside near the predicted binding site of H-NOX-associated cyclic-di-GMP synthase/phosphodiesterase, the signaling partner for Sw H-NOX (29). Significant shifts were also observed for residues E27 and E57, near the αA/αD interface. Though conserved, IWP-051 binding to Sw H-NOX is weaker than to Ms sGC-NT, with $K_d = 1.9$ mM estimated for the CO complex by chemical shift perturbation NMR titration (Fig. 5B, supplemental Table S6) compared with $K_d$ values of 0.03-3.8 μM for various stimulator compounds binding to the CO complexes of Ms sGC-NT constructs (20).

**Molecular modeling of stimulator binding to sGC—**Our labeling and NMR data indicate the functional binding site for stimulator compounds resides in the β1 H-NOX domain. While a high-resolution structure of an sGC H-NOX domain has
not been reported, crystal structures for several bacterial homologues are known, including those from *Caldanaerobacter subterraneus* (30), also known as *Thermoanaerobacter tengcongensis* (22,31), *Nostoc* sp. 7120 (32,33) and *Shewanella oneidensis* (34). These structures display the same overall fold and provide a solid scaffold for understanding H-NOX structure in sGC function (reviewed in (4,5,23)). The overall H-NOX fold is ~180 residues long and displays an N-terminal sub-domain encompassing residues 1-60, which is dominated by a 3-helix bundle, followed by a larger mixed helix-sheet sub-domain that contains the heme pocket. Alignment of the larger sub-domains of several H-NOX structures indicate the smaller and larger domains can move independently of one another, altering the orientation of the two domains, which has been proposed to be key for signal transduction by H-NOX domains and proteins (29,30,32).

Most of the residues implicated in compound binding by photoaffinity labeling and NMR lie at the interface of the H-NOX sub-domains (Fig. 5A), making this interface an intriguing possibility for stimulator binding. While the large domain includes the heme-binding pocket, the small domain covers the heme distal pocket and contacts the heme edge. It is easy to imagine that changes in the sub-domain interface would affect heme properties, ligand affinity and signal transduction. Thus, our working hypothesis is that stimulator compounds bind in this region and we have modeled IWP-854 binding into a pocket at the sub-domain interface (Fig. 6A).

Modeling of compound into heterodimeric sGC was more challenging since atomic-level models are unavailable. For this we utilized a previously described model for *Ms* sGC-NT based on small-angle X-ray scattering (SAXS), chemical cross-linking and homology modeling of the H-NOX, PAS and coiled-coil domains (19). The labels found most frequently in the present study were to the coiled-coil. Encouragingly, these residues in the coiled-coil lie near the labeled residues in the αA, αC, and αD helixes of the β1 H-NOX domain (Fig. 6B).

**Mutational analysis of the proposed stimulator binding site**—Mutations designed to block compound access were introduced into the β1 H-NOX subdomain interface. Three residues predicted to occupy different regions of the binding site, Leu 12, Thr 48 and Ile 66, were targeted for mutation and changed to tryptophan. Mutations were originally generated in *Ms* sGC-NT23, which lacks the α1 H-NOX domain (Fig. 1A); however, these constructs led to insoluble protein. We then turned to *Ms* sGC-NT25, which resembles *Ms* sGC-NT13 but is extended by 9 residues at the C-terminal end of both α1 and β1 subunits. Mutations L12W, T48W, L66W and the double mutation L12W/T48W were engineered into *Ms* sGC-NT25, successfully purified and examined for CO binding affinity in the presence or absence of BAY 41-2272 (Table 2).

The T48W single mutation resulted in ~3-fold weaker binding affinity for CO in the absence of BAY 41-2272 (Table 2). No appreciable changes in CO binding affinity were observed for the L12W and I66W single mutations, while the L12W/T48W double mutant bound CO ~2-fold weaker than wild type. These data indicate the mutations had minimal effect on the heme environment.

In contrast, stimulation by BAY 41-2272 was reduced in all of the mutated proteins, quite dramatically in two cases. Addition of 10 µM BAY 41-2272 to wild-type protein increased CO binding affinity by 65-fold, as expected. Single mutations T48W and I66W led to proteins with moderately less response to BAY 41-2272, while mutation L12W displayed only 13-fold enhancement by stimulator, and double mutant L12W/T48W lost nearly all response, displaying only 7-fold enhancement in the presence of BAY 41-2272, 9-fold worse than wild type.

**DISCUSSION**

sGC stimulators were first discovered over 20 years ago and have since been improved such that one compound is in clinical use and several more are likely to follow. Yet understanding where these compounds bind and how they function has lagged behind, potentially hampering discovery of novel compounds with enhanced pharmacological properties. Here, we resolve the longstanding question of where stimulators bind, narrowing the binding site to the heme domain where NO binding stimulates catalytic activity. The stimulator binding site is apparently evolutionarily conserved and found in H-NOX domains since they first appeared in bacteria.
In the present study, we used a photoactivatable stimulator compound coupled with LC-MS/MS, along with TrNOESY and chemical shift perturbation NMR, to narrow the binding site to the β1 H-NOX domain. No binding was observed to the cyclase domain active site or to the pseudosymmetric site, nor was there binding to the α1 H-NOX domain, as suggested by a previous labeling study (35). This discrepancy likely results from choice of labeling reagent, with the present study utilizing a diazirine versus an aryl azide in the former study. Diazirines improve upon aryl azides with quicker reaction times and a lower frequency of stable intermediates that are capable of diffusing away from the binding site (15), which in the case of aryl azides, includes ketenimine decay products that react strongly with nucleophiles such as cysteines. Since both residues previously identified were cysteines (35), the reaction may have been with the ketenimine.

We modeled a possible binding complex in which stimulators bind at the interface of the two H-NOX subdomains, where most of the residues with labeling and NMR chemical shift peaks were located (Fig. 6A). This pocket was previously identified as part of a tunnel suggested to be of importance for NO, CO and O₂ gas exchange with the heme distal pocket in bacterial H-NOX proteins (33,34). Filling this pocket with compound provides a possible mechanism for stimulation and may explain the conservation of binding in H-NOX proteins. With the most critical portions of stimulator compounds filling the gas-exchange tunnel, we modeled the IWP-854 pyrimidine ring, which contains the photoactive diazirine, to be near major H-NOX and coiled-coil labeled peptides, using our previously modeled domain arrangement (Fig. 6B) (19). Since the coiled-coil was labeled in all experiments in which it was present, these results suggest a compact domain arrangement of sGC is likely to occur in high abundance.

To test our model, we introduced a series of mutations into the proposed binding pocket (Table 2). All three mutations led to proteins with reduced response to stimulator while retaining similar CO binding affinity as wild-type protein in the absence of compound. Double mutant L12W/T48W displayed the lowest stimulator response, yielding only a 9-fold reduction in CO enhancement compared to wild type. While it is possible that general allosteric response is altered in the mutated proteins, it is more likely that the L12W and T48W mutations physically block stimulator binding or distort the pocket such that binding affinity is lost.

How do stimulator compounds stimulate? The answer to this question is still unknown but key factors are becoming apparent (recently reviewed in (4,5)). One key feature of stimulator binding is its ability to enhance affinity for NO and CO to the sGC heme, which may in turn enhance catalysis. Increased NO/CO affinity is due in part to increased geminate recombination, a process in which trapping dissociated gas molecules in the protein favors rebinding to heme over escape into bulk solution (17,36). A variety of mechanisms might contribute to increased geminate recombination upon stimulator binding, including induction of a heme conformation with enhanced ligand binding on-rates or the blocking of ligand escape paths. The sGC β1 H-NOX domain has inherently high affinity for NO and CO in the isolated state. This affinity is dampened as additional domains are included in the protein, with addition of α1 pseudo H-NOX, α1 PAS and the full-length coiled-coil each serving to lower heme affinity for gaseous ligands (19,20). Binding of stimulator partially overcomes the inhibitory effect of additional sGC domains, possibly through release of domain contacts or through direct binding to a high-affinity H-NOX domain conformation, or both. Here, we show that stimulator binding is not only directly to the H-NOX domain but may also plug a proposed tunnel implicated in gas molecule release from the distal pocket. Additionally, analysis of bacterial H-NOX proteins suggests movements in the N- and C-terminal subdomains are intimately connected to heme geometry and ligand binding affinity (23).

A second key feature for stimulator activity is its linked equilibria with NO and CO binding. Binding of NO or CO leads to an sGC conformation with higher affinity for stimulator compound just as stimulator binding leads to higher affinity for NO and CO. The simplest model for explaining such linked equilibria is through allostery: there is an H-NOX conformation that binds both gaseous ligand and stimulator with high affinity, and binding of either stimulator or NO/CO induces this conformation.
Finally, there is a linked equilibrium between affinity of NO at the heme and GTP at the active site. Binding of NO leads to a cyclase conformation with higher GTP affinity, lowering $K_m$ for catalysis, and also increasing $V_{max}$. Binding of stimulator has the same effect. Binding of nucleotide alters NO affinity as one would expect from linked equilibria, although the details of this are complicated and may involve more than one nucleotide binding site. Presumably, the affinity of stimulator for full-length sGC would also increase in the presence nucleotide, but this has not yet been examined.

In summary, we have shown that stimulators bind to the H-NOX domain of sGC as well as to bacterial homologs. Binding likely occurs at the interface of the H-NOX large and small subdomains, and may act through both inducing an active conformation and through directly blocking a tunnel for gas release to bulk solvent. These data provide insight into sGC function and stimulator action, and provide a roadmap for improved compounds targeting disease.

**EXPERIMENTAL PROCEDURES**

**Materials**—Chemicals were purchased from Sigma Aldrich (St. Louis, MO) unless otherwise indicated. Uniformly labeled $^{15}$N-ammonium chloride, $^{13}$C-glucose and deuterium oxide (D$_2$O) were purchased from Cambridge Isotope Laboratories. 2-(N,N-Diethylamino)diazelenolate-2-oxide (DEA/NO) was generously provided by Dr. Katrina Miranda (University of Arizona). Full-length human $\alpha_1\beta_1$ sGC expressed in SF9 cells with C-terminal Histag was purchased from Enzo Life Sciences, Inc. (Farmingdale, NY). HEK293T cells were acquired from the American Type Culture Collection (ATCC). Turbofekt was purchased from Fermentas. DMEM media was purchased from Gibco Life Technologies (Waltham, MA). Fetal bovine serum was obtained from the University of Arizona Cancer Center (Tucson, AZ). Sequencing grade trypsin and chymotrypsin were acquired from Promega (Madison, WI), and C18 zip-tips were purchased from Pierce Thermo Fisher Scientific (Waltham, MA).

**Syntheses**—IWP-051 and IWP-898 (identical to phosphodiesterase 9A inhibitor PF-044447943) were produced as previously described (11,28). Photoactive labeling reagent IWP-854 was synthesized and purified in a manner similar to IWP-051. Detailed synthetic procedures for IWP-854 are included in the supplemental materials.

**Generation of sGC and bacterial H-NOX constructs**—Construction of Ms sGC-NT13 ($\alpha_1$ His$_S$-49-450, $\beta_1$ 1-380) and Ms sGC-NT21 ($\alpha_1$ His$_S$-272-450, $\beta_1$ 1-380, Fig. 1A) were previously described (18-20). Plasmid pETDuet-1-NT25, coding for Ms sGC-NT25 ($\alpha_1$ His$_S$-49-459-Strep, $\beta_1$ 1-389), was generated by amplifying cDNA coding for $\alpha_1$ 49-459 by PCR using primers F1 and R1 (primer sequences are in supplemental Table S1). PCR product was subcloned into vector pGEM-T (Promega Corporation, Madison, WI), then cloned into plasmid pETDuet-1-NT2 following the removal of the original $\alpha_1$ subunit with restriction enzymes BamHI and NotI. A stop codon (TAA) was inserted into the Ms sGC $\beta_1$ subunit after Leu 389 using the QuikChange Lightning Site-Directed Mutagenesis Kit (Agilent Technologies, Santa Clara, CA) with primers F2 and R2. Mutations were introduced into pETDuet-1-NT25 coding for Ms sGC-NT25 using the Quikchange Lightning Site-Directed Mutagenesis Kit and primers F6-F8 and R6-R8.

To generate Ms sGC-NT23 ($\alpha_1$ His$_S$-272-459-Strep, $\beta_1$ 1-389), an equimolar mixture of oligonucleotides F3 and R3 (supplemental Table S1) were incubated at 95 °C for 2 min and cooled to room temperature, creating a 37 base double-stranded DNA with 4-base overhangs on each end. The resulting product was ligated into plasmid pETDuet-1-NT25 with T4 ligase following removal of the $\alpha_1$ H-NOX domain with BamHI and Nhel restriction enzymes.

Constructs coding for full length human sGC were generated by amplifying cDNA coding for sequences for $\alpha_1$ 1-690-Strep-tag II and $\beta_1$ 1-619-His$_S$ using primers F4, R4, F5 and R5. PCR product for Hs sGC $\alpha_1$ was cloned into plasmid pCMV_3TAG9 between the restriction sites BamHI and HindIII to create plasmid pCMV_3TAG9_sGCa1. PCR products for Hs sGC $\beta_1$ was cloned into plasmid pCMV_3TAG3A between the restriction sites SacI and Xhol to create plasmid pCMV_3TAG3A_sGC$\beta_1$. 
Genes for H-NOX proteins from *Nostoc sp. PCC 7120* (Ns H-NOX; residues 1-182; NCBI Ref Seq: WP_010996435.1), *Shewanella oneidensis* (So H-NOX; residues 1-181; NCBI Ref Seq: WP_011072197.1), *Shewanella woodyi* (Sw H-NOX; residues 1-182; NCBI Ref Seq: WP_012325363.1), and *Clostridium botulinum* (Cb SONO; residues 1-186; NCBI Ref Seq: WP_011072197.1) were synthesized, cloned into plasmid pET21b+ between restriction sites NdeI and XhoI (GenScript Biotech Corporation). All constructs contain a C-terminal TEV cleavage sequence and a His6 affinity tag (ENLYFQSLEHDEHHHHH).

**Expression and Purification of Ms sGC-NT and Bacterial H-NOX—Ms sGC-NT13, Ms sGC-NT21, Ms sGC-NT23 and Ms sGC-NT25 were expressed and purified from *Escherichia coli* as previously described (18-20). Ms sGC-NT21 β1 (1-380) was isolated from Ms sGC-NT21 by washing the column-bound sample with CO-saturated buffer, resulting in selective elution of the β1 subunit.

Plasmids coding for Ns H-NOX, So H-NOX, or Cb SONO were transformed into Rosetta competent cells in Terrific Broth media and grown at 37 °C while shaking at 225 rpm. Once an OD600 of 0.8-1.0 was reached, protein expression was initiated by adding 0.1 mM δ-aminolevulinic acid (ALA) and 0.5 mM IPTG. Expression continued for 18-22 hours at 16 °C and cells were harvested by centrifugation at 4500 RPM with a JLA-8.1000 rotor (Beckman Coulter) for 20 minutes at 4 °C. Pellets were flash frozen in liquid nitrogen and stored at -80 °C.

Ns H-NOX, So H-NOX, and Cb SONO were purified by suspending cells in buffer B (50 mM sodium phosphate, pH 7.4, 300 mM NaCl) supplemented with 0.75 mM DNase I and 1 mM PMSF. Cells were lysed by French press and debris was removed by ultracentrifugation at 40,000 rpm in a Ti45 rotor for 35 min at 4 °C. The supernatant was loaded onto a HisTrap FF NiNTA affinity column (GE Healthcare) and sample was extensively washed with buffer B. Protein was eluted by supplementing buffer B with 30 mM EDTA, followed by buffer exchange into 50 mM sodium phosphate, pH 7.5, 90 mM NaCl, <0.3 mM EDTA using centrifugal filters. The His6 affinity-tag was removed by incubation with a 1:100 (protease:protein) molar ratio of TEV protease, prepared as previously described (20), overnight at 4 °C. Sample was passed over a HisTrap FF column and cleaved protein was further purified using a Superdex 200 gel filtration column equilibrated with buffer D (20 mM Tris-HCl, pH 7.4, 100 mM NaCl). Protein concentrations were assessed based on Soret band absorption and samples were frozen in liquid nitrogen for storage at -80 °C.

Sw H-NOX was expressed and purified in the same manner as other H-NOX proteins with the following exceptions: For unlabelled Sw H-NOX, plasmids were transformed into *Escherichia coli* Tuner (DE3) pLysS competent cells and grown in M9 media. Expression was induced with 0.05 mM ALA and 0.1 mM IPTG. Isotopically labeled Sw H-NOX was grown in M9 media isotopically enriched for 15N or 13C,15N and protein expression induced with 0.5 mM ALA and 0.5 mM IPTG. All buffers used to purify labeled Sw H-NOX were extensively degassed and supplemented with 0.5 mM TCEP. Once loaded onto the HisTrap FF column, protein was washed with buffer C (50 mM sodium phosphate, pH 7.5, 500 mM NaCl, 20 mM EDTA) and eluted by supplementing buffer C with 60 mM EDTA. Sample concentration was measured with a Pierce 660nm Protein Assay (Thermo Scientific).

**Determination of CO dissociation constants**—CO titrations were measured using a Cary350 UV/visible spectrophotometer (Agilent Technologies, Santa Clara, CA) and a cuvette with 10 cm pathlength. *Kd* CO measurements were determined as previously described (19,20), with the exception that samples were suspended in buffer A, and BAY 41-2272 was solubilized with a final concentration of 5% EtOH.

**Expression of human sGC—HEK293T cells** were grown in Dulbecco's Modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) at 37 °C with 5% CO2. A transfection mixture containing 22.5 μg pCMV_3TAG9_sGCα1, 2.5 μg pCMV_3TAG3A_sGC β1, and 31.25 μL Turbofect (Thermo Fischer Scientific) was assembled in 2.5 mL serum-free DMEM. The transfection mixture was incubated for 30 min at room temperature and added drop wise to a 10 cm dish containing HEK293T cells grown to 65-80% confluency. Protein expression continued for 24 h at 37 °C with 5% CO2.
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cGMP measurement—HEK293T cells transfected with human sGC were washed twice with PBS and suspended in buffer A supplemented with 8 mM MgCl₂, 0.5 mM IBMX, 1 mM PMSF, and 1:100 dilution of protease inhibitor cocktail (Sigma). Cells were lysed with 30 strokes of a 25-gauge needle and debris was removed by centrifugation at 16,000 g for 20 min at 4 °C. Lysate was combined with IWP-854 or BAY 41-2272 (5 µM) and DEA/NO (100 µM) as indicated. Samples were incubated for 10 min at room temperature prior to adding GTP (1 mM). Reactions proceeded for 5 min at 37 °C and were quenched with 1.5% glacial acetic acid. Precipitated protein was removed by centrifugation at 16,000 g for 10 min at 4 °C and the supernatant was diluted 1:100 in 50 mM sodium phosphate (pH 7.0), 0.2% BSA, 0.2% Na₂SO₄. cGMP was quantified using a commercially available homogenous time resolved fluorescence (HTRF) assay according to the manufacturer’s instructions (CisBio). All samples were measured in duplicate using a Synergy H1 fluorescent plate reader (BioTek). HTRF measurements were analyzed according to the manufacturer’s instructions using Sigma Plot (Systat Software, Inc.).

Photoaffinity labeling sGC and bacterial H-NOX with IWP-854—The sGC or bacterial H-NOX construct of interest (1 µM for sGC, 10 µM for bacterial H-NOX) were suspended in buffer A. DEA/NO (100 µM), CO (50 µM), and varying concentrations of BAY 41-2272 (1-100 µM) were added as indicated. For competition assays, a final concentration of 5% ethanol was added to maintain the solubility of BAY 41-2272. Samples were equilibrated for 10 min at room temperature before adding IWP-854 (1 µM for sGC, 10 µM for bacterial H-NOX). Samples were incubated for 10 min at room temperature in the dark, placed in a 96-well tissue culture plate on ice, and irradiated for 20 minutes with broad band UV light (366 nm maximum) using a multiband UVGL-58 mineralight lamp (UVP, Upland, CA).

Detecting photoaffinity labeling by western blot—Samples were suspended in 1x SDS loading buffer and 1 µg protein was run on a 15% bis-acrylamide gel for 90 minutes at 96 V. Protein was transferred to a nitrocellulose membrane at 100 V for 1 h at 4 °C. The membrane was blocked for 1 h in 5% BSA in PBS-T (0.1% Tween-20) and incubated in a 1:1000 dilution of primary antibody (Cell Signal #5597 for biotin, Abcam ab76949 for Strept-tag II, QED Biosciences #18814-01 for His₅) over night at 4 °C. The membrane was washed three times in PBS-T and incubated in a 1:10000 dilution of secondary antibody (Li-Cor #925-68071) for 2 h while shaking at room temperature. Membranes were washed an additional three times and imaged using the Odyssey Infrared Imaging System.

Western blots involving Hs sGC were performed in a similar manner with the following exceptions: A total of 200 ng protein were run on NuPAGE 4-12% Bis-Tris gel (Invitrogen). The biotin-affinity tag was detected with a 1:2000 dilution of IRdye800 streptavidin (Li-Cor cat # C40403-1). A 1:1000 dilution of primary antibody (Abcam ab154841 for Hs sGC β1 subunit) and a 1:15000 dilution of secondary antibody (Li-Cor #926-68071) were used as a loading control.

Preparing samples for mass spectrometry—Photoaffinity labeled samples were buffer exchanged into 100 mM ammonium bicarbonate (pH 8.0) using 10 kDa centrifugal filters (Amicon). Samples were reduced with 12 mM dithiothreitol for 45 min at 56 °C, alkylated with 20 mM iodoacetic acid for 30 min at room temperature in the dark, and digested with a 1:30 (protease:protein) ratio of trypsin or chymotrypsin overnight at 37 °C or 30 °C, respectively. Samples digested with chymotrypsin contained 1 mM CaCl₂. Digested peptides were cleaned using C18 zip tips (Pierce Thermo Fisher Scientific), dried by speedvac and stored in -20 °C.

LC-MS/MS analysis of photoaffinity labeled samples—Digested peptides were analyzed by LC-MS/MS using a LTQ Velos Orbitrap mass spectrometer (Thermo Fischer Scientific). Peptides were eluted from a C18 pre-column (100 µm i.d. x 2 cm, Thermo Fisher Scientific) onto an analytical column (75 µm i.d. x 2 cm, Thermo Fisher Scientific). Solvent A was 0.1% FA. Solvent B (ACN, 0.1% FA) was applied as follows: 5-20% B over 75 min, 20-35% B over 25 min, 35% B for 19 min, 3 min ramp to 95% B and held for 18 min. Flow rates were 400 nL/min directed to an Advin NanoMate nano-ESI source (Advion, Ithaca, NY) held at 1.75 eV applied voltage.

Data-dependent scanning was performed with Xcalibur v 2.1.0 software using a survey mass scan at 60,000 resolution in the Orbitrap.
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Analyzer scanning mass/charge (m/z) range of 350-1600, followed by collision-induced dissociation (CID) tandem mass spectrometry (MS/MS) of the 6 most intense ions in the Orbitrap analyzer at 7,500 resolution. Precursor ions were selected by the monoisotopic precursor selection (MPS) setting, with the instrument set to observe fragment ions once and then excluded from analysis for 45 seconds, allowing for interrogation of lower abundance ions. Ions were excluded with a ±10 ppm window. Ionization of IWP-854 increases the charge state of the peptide, allowing for increased selection of labeled peptides by excluding precursor ions with a charge less than +3.

Tandem mass spectra were searched against a protein database containing 5200 entries, including sequences for all sGC and H-NOX constructs analyzed, the proteome for Escherichia coli BL21, and common contaminants. Searches were performed using Thermo Proteome Discoverer 1.3, version 1.3.0.339 (Thermo Fischer Scientific) considering the tryptic peptides with up to 2 missed cleavage sites. Iodacetamide derivatives of cysteines and oxidation of methionines were specified as variable modifications. Modification by IWP-854 (1422.717 Da) was searched against all 20 amino acid residues in an iterative fashion. The presence of IWP-854 was confirmed by a peak corresponding to the mass of the precursor ion minus m/z 270.127 Da (Fig. 3A). All labeled peptides were initially identified automatically by the Discoverer software. Following initial identification, manual assignment of peptides was performed as necessary based on precursor mass, MS/MS spectra, and column retention time.

**NMR sample preparation**—The diamagnetic Sw H-NOX Fe(II)-CO complex was used in backbone assignment and chemical shift perturbation experiments. To prepare the sample, the isotopically labeled Sw H-NOX was treated with 10 mM dithionite in an extensively degassed buffer containing 50 mM Tris-HCl (pH 8.0), 50 mM NaCl, and then saturated with CO in a sealed tube. Dithionite was removed by buffer-exchange to an extensively degassed, then CO-saturated buffer containing 50 mM sodium/potassium phosphate (pH 7.5), 50 mM NaCl, and 10% D$_2$O for NMR measurement. NMR experiments were performed in a CO-saturated and sealed 5.0 mm NMR tube. The concentration of NMR samples was ~0.8 mM for backbone assignment. An $^{15}$N-HSQC spectrum was collected before and after each experiment to evaluate the stability of the protein. IWP-051 and PDE9A inhibitor were prepared by dissolving the compounds into DMSO-d$_6$ (99.9 atom % D, Aldrich) to a final concentration of 25 mM. 2,2-dimethyl-2-silapentane-5-sulfonate (DSS) was included as an internal reference in the TrNOESY sample and used to calibrate the concentration of IWP-051 compound.

**NMR spectroscopy and data analysis**—NMR experiments for protein backbone resonance assignment were carried out on Agilent 18.8 T NMR spectrometer equipped with a triple resonance cryogenic probe at National Magnetic Resonance Facility at Madison. NMR experiments for CSP and TrNOESY were carried out on Agilent 14.0 T spectrometer equipped with a triple resonance cryogenic probe at University of Arizona. All experiments except for TrNOESY were performed at 20°C. TrNOESY experiments were performed at 25°C. NMR data were processed using NMRPipe (37) and in combination with MddNMR (38,39) or SMILE (40) for non-uniformly sampled data. The NMRFAM-SPARKY (41), PINE (42) and PINE-SPARKY (43) were used for resonance assignment and spectrum analysis.

**Protein backbone resonance assignment**—All backbone and side-chain resonance experiments were acquired using 40-50% non-uniformly sampling(38,39)(40). Sequence-specific backbone resonance assignments were determined using the following triple resonance experiments: 3D-HNCA (44), 3D-HN(CO)(CA), 3D-HNCACB (44), 3D-CB(CA)(CO)NH, 3D-HNCO, and 3D-(HCA)(CO)(CA)NH. Side-chain resonance experiments including 3D-(C(CO)NH (45), 3D-H(CCO)NH (45), 3D-HCCH-TOCSY and 3D $^{15}$N-NOESY-HSQC (100-ms mixing time) (46) were used to verify the backbone resonance assignments. More than 90% of residues were assigned.

**Chemical shift perturbation**—Chemical shift perturbations (CSPs) were measured using 336 µM $^{15}$N-labeled Sw H-NOX with increasing concentration of IWP-051 (100 µM to 4000 µM) or PDE9A inhibitor (200 µM to 1600 µM). Residues were identified using the assigned $^{1}$H-$^{15}$N
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HSQC spectrum. The magnitude of CSP for each residue was calculated using the following equation (47):

$$\Delta \delta = \frac{\sqrt{(\Delta \delta H)^2 + (\Delta \delta N/5)^2}}{2}$$  \hspace{1cm} (1)

In which $\Delta \delta H$ is the change in $^1$H shifts and $\Delta \delta N$ is the change in $^{15}$N shifts in ppm. A scaling factor of 0.2 was applied to the $^{15}$N shifts. Control experiments titrating only DMSO-d6 into the protein were used to correct the solvent perturbations from DMSO-d6. For residues with significant perturbations, their CSPs were plotted as a function of ligand concentration and fitted into the following equation (48):

$$\Delta \delta = \left(\frac{\Delta \delta_{\text{max}}}{2[P_L]}\right) \times \left[ [K_d + [P_L] + [L] - \sqrt{(K_d + [P_L] + [L])^2 - 4[P_L][L]} \right]$$  \hspace{1cm} (2)

In which $\Delta \delta$ is the adjusted chemical shift change, $\Delta \delta_{\text{max}}$ is the maximum adjusted chemical shift change at saturation, $[P_L]$ is total protein concentration, $[L]$ is the ligand concentration, and $K_d$ is the equilibrium dissociation constant. $K_d$ was extracted by fitting the data using xcrvfit.

TrNOESY measurements—The hydrogen chemical shifts of IWP-051 were assigned as previously described (11) and confirmed by 2D-COSY, 2D-TOCSY, and 2D-NOESY experiments for samples dissolved in D$_2$O. Transferred nuclear Overhauser effect spectroscopy (TrNOESY) experiments were performed with IWP-051 or PDE9A inhibitor dissolved in 99.9% D$_2$O, 50 mM sodium phosphate (pH 7.5), 100 mM NaCl, 4% DMSO-d6, 2 mM dithionite and 100 µM DSS. Protein/ligand molar ratios of 1/50 for Ms sGC-NT23, 1/29 for Cb SONO and 1/19 for Sw H-NOX were used with IWP-051 and ratios of 1/31 for Ms sGC-NT23 was used with PDE9A inhibitor. For Cb SONO, a pH of 8.0 was used to increase the stability of the protein. A conventional NOESY sequence was used with 256 x 2048 data matrices and with presaturation for water suppression. TrNOESY-derived distances were calibrated using the distance between H9 and H10 from the modeled IWP-051 structure. The NOE contribution from H(11) was scaled by a factor of 2 prior to distance calculation to account for the two equivalent hydrogens on the methylene group.

Molecular modeling—A molecular model for Ms sGC-NT13 was previously assembled using small-angle X-ray scattering (SAXS), chemical cross-linking and domain homology modeling (19). Models for compounds IWP-051 and IWP-854 were prepared by first generating a SMILES string in ChemDraw (PerkinElmer Informatics, Inc.) and submitting the string to the Grade Web Server (http://grade.globalphasing.org/cgi-bin/grade/server.cgi), which generates energy-minimized coordinates and refinement parameters using known structures in the Cambridge Structural Database. Modeling of compound binding was done manually in COOT (49) followed by energy minimization in REFMAC5 (50) as encoded in CCP4i (51). Structure figures were prepared using the PyMOL Molecular Graphics System, Version 1.8.6.0 Schrödinger, LLC.

Data availability—All data that support the findings of this study are in the published article (and its supplementary information files) or are available from the corresponding author upon reasonable request.

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Conflict of Interest: The authors declare the following competing financial interests: S.G.B., J.E.S., R.S., T.K., P.A.R. and J.J. are employees of Ironwood Pharmaceuticals and developing sGC target compounds for marketing. They own stock or stock options in Ironwood Pharmaceuticals, Inc. W.R.M. has received funding by Ironwood Pharmaceuticals for study of novel sGC stimulators.

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Stimulator binding to the sGC β1 H-NOX domain

Figure Legends

FIGURE 1. sGC constructs and stimulators. (A) Diagram highlighting the domain structure of sGC constructs included in the present study. In addition to full-length human sGC, several truncated versions of Manduca sGC were examined, including Ms sGC-NT13 (α1 49-450, β1 1-380), Ms sGC-NT25 (α1 49-459, β1 1-389), Ms sGC-NT23 (α1 272-459, β1 1-389) and Ms sGC-β1 (β1 1-380), which may be homodimeric. Bacterial H-NOX proteins from Nostoc sp. PCC 7120 (1-182), Shewanella oneidensis (1-181), Shewanella woodyi (1-182), and Clostridium botulinum (1-186) were also examined. (B) Chemical structures for IWP-854, IWP-051 and BAY 41-2272. The fragmentation position for IWP-854 during mass spectrometry is indicated (270.127).

FIGURE 2. IWP-854 labeling is specific to the stimulator binding site. (A) Catalytic activity for human sGC in the absence or presence of DEA/NO (100 µM) and stimulator (5 µM). sGC α and β genes were transiently transfected into HEK293T cells and activity measured after cell lysis. Error bars represent the average and standard deviation for three independent measurements (panels A and B). (B) Saturation curves for CO binding to Ms sGC-NT23 (30 nM) in the absence or presence of stimulator (5 µM). (C) Representative western blot illustrating IWP-854 labeling of Ms sGC-NT23. Selective linkage of IWP-854 (1 µM) to the β1 subunit of Ms sGC-NT23 (1 µM) is readily visualized with an anti-biotin antibody after 15 minutes of irradiation with 350-365 nm UV light (MW β1 ~45 kDa). Labeling of α1 (MW ~20 kDa) is not observed. Addition of BAY 41-2272 (1-25 µM) competes away IWP-854 in a concentration-dependent manner. Loading control: anti-Strep-affinity tag (Ms sGC-NT23 α1). (D) Similar to panel (C) using full-length recombinant Hs sGC (1 µM; MW α1 ~70 kDa, MW β1 ~86 kDa), IWP-854 (1 µM), and BAY 41-2272 (1-50 µM). Loading control: anti-Hs sGC β1. (E) Similar to panel (C) using Cb SONO (10 µM, MW ~23 kDa), IWP-854 (10 µM), and BAY 41-2272 (10-50 µM). Cb SONO was reduced to the ferrous state (Soret 431 nm) with 2 mM dithionite and saturated with CO (100 µM). Dithionite was removed with a desalting spin-column prior to stimulator addition. Loading control: anti-His6-affinity tag.

FIGURE 3. Mass spectrum of an Ms sGC-NT23 peptide modified by IWP-854. (As) LC-MS/MS spectrum of peptide β1 1-15 modified by IWP-854, undertaken in high resolution/high resolution mode. Loss of the protonated biotin-containing fragment is clearly indicated (m/z = 270.127) along with the z = +3 labeled peptide (m/z = 993.181). Chemical structures for the biotin-containing fragment (m/z = 270.127) and the target peptide modified by IWP-854 (m/z = 993.181) are depicted in the figure. Nearly all possible b and y ions were observed. Labeling was to Tyr 7. (B) Arrangement of labeled residues in a model for Ms sGC-NT (α1 49-469, β1 1-399), generated through homology modeling, SAX analysis and chemical cross-linking (19). The subdomains of the β1 H-NOX domain are shown in green (N-terminal subdomain) and light blue (C-terminal subdomain). The remainder of the β1 subunit is depicted in tan and the α1 subunit is shown in light gray. Modified residues are labeled and shown in red.

FIGURE 4. Transferred NOE NMR spectroscopy. (A) Chemical structure of IWP-051 with atoms numbered as in panel B. (B) TrNOESY spectra for free IWP-051 (upper panel; 292 µM, mixing time 1,600 ms) and IWP-051 (292 µM) bound to Ms sGC-NT23 (lower panel; 5.3 µM, mixing time 400 ms). NOE cross peak intensity relative to the negative diagonal peaks (black) change from positive (free IWP-051, blue) to negative (protein bound, black), upon binding. Three new peaks appear (11H-9H, 11H-2H, 11H-14H) and one peak is lost (20H-10H) on binding, indicating IWP-051 adopts a single primary conformation. Missing peaks are indicated with red circles (left panel) and blue box (right panel).
FIGURE 5. Chemical shift perturbation NMR spectroscopy. (A) Surface and cartoon representation for the modeled structure of Ms sGC-NT23 bound to IWP-051. Residues with large $\Delta \delta$ in Sw H-NOX are colored blue (helix $\alpha$A), red (helix $\alpha$D) and purple (backside), labeled according to the Sw H-NOX sequence. IWP-051 (atom colors) and heme (yellow) are shown as sticks. (B) Prominent $\Delta \delta$ upon titration of IWP-051 into Sw H-NOX (336 $\mu$M), fitted to equation 2 (left). Superimposed $^1$H-$^15$N HSQC spectra of increasing concentration of IWP-051 (red to blue) are shown for each residue (right). The shift along the titration indicates fast exchange of ligand binding. (C) Shown are chemical shift perturbations ($\Delta \delta$) in Sw H-NOX (336 $\mu$M) induced by IWP-051 (1.6 mM, blue) or PDE9A inhibitor (1.6 mM, red, negative control), after subtraction of perturbations from DMSO alone. Residues with larger changes in $\Delta \delta$ (>3$\sigma$, blue line) are labeled. Secondary structure is indicated with cylinders (helices A-G) and arrows (beta sheet strands 1-4).

FIGURE 6. Molecular model of modified residues and proposed stimulator binding pocket. (A) Model for the $\beta$1 H-NOX domain of sGC with bound labeling compound. IWP-854 binding is proposed to be in the pocket formed between subdomains. The N-terminal subdomain is shown in green and the C-terminal subdomain in light blue. IWP-854 is depicted as sticks (carbon light gray, nitrogen blue, oxygen red) with biotin and the PEG linker left out for clarity. Heme is shown as black sticks. Residues prominently labeled by IWP-0854 in sGC and bacterial H-NOX proteins are shown in dark blue. Residues with prominent chemical shift perturbations in Sw H-NOX are shown in red. (B) Surface representation for Ms sGC-NT highlighting the proposed binding pocket. Colors for the $\beta$1 H-NOX are the same as in panel (A). The remaining protein is shown in light gray.
| Modified Residues | Construct | Occurance | Error |
|------------------|-----------|-----------|-------|
| **H-NOX Domain** |           |           |       |
| β1 6-9<sup>e</sup> | Ms sGC-NT<sup>d</sup> (6-8)<sup>f</sup> | 18/26 | 3.2 ± 2.0 |
|                   | Sw H-NOX (7-9)<sup>f</sup> | 2/2 | 0.8 ± 0.8 |
|                   | Cb SONO (7)<sup>f</sup> | 3/3 | 1.8 ± 0.1 |
| β1 41-46<sup>e</sup> | Hs sGC (41-44)<sup>f</sup> | 1/2 | 1.4 |
|                   | So H-NOX (42-46)<sup>f</sup> | 4/4 | 1.5 ± 2.3 |
|                   | Ns H-NOX (44-46)<sup>f</sup> | 3/3 | 0.4 ± 0.1 |
| β1 47-52<sup>e</sup> | Hs sGC (48-51)<sup>f</sup> | 2/2 | 2.1 ± 1.0 |
|                   | Ns H-NOX (50-52)<sup>f</sup> | 3/3 | 1.1 ± 0.7 |
|                   | Sw H-NOX (47-49)<sup>f</sup> | 2/2 | 2.1 ± 0.4 |
| β1 76-84<sup>e</sup> | Ns H-NOX (79-84)<sup>f</sup> | 2/3 | 1.3 ± 1.0 |
|                   | Sw H-NOX (76-81)<sup>f</sup> | 2/2 | 3.5 ± 0.1 |
|                   | Cb SONO (76-78)<sup>f</sup> | 3/3 | 1.1 ± 0.1 |
| **Linker (H-NOX Domain – PAS Domain)** |           |           |       |
| β1 195-198 | Ms sGC-NT<sup>d</sup> | 11/26 | 2.4 ± 1.0 |
| **Linker (PAS Domain – CC Domain)** |           |           |       |
| β1 328-331 | Ms sGC-NT<sup>d</sup> | 11/26 | 3.1 ± 1.7 |
| **CC Domain** |           |           |       |
| β1 361-362 | Ms sGC-NT<sup>d</sup> | 26/26 | 2.6 ± 1.7 |
| β1 365-366 | Ms sGC-NT<sup>d</sup> | 26/26 | 2.6 ± 1.7 |
| β1 370-371 | Hs sGC | 2/2 | 1.4 ± 0.3 |
| β1 374-375 | Hs sGC | 2/2 | 1.4 ± 0.3 |
| β1 385 | Hs sGC | 2/2 | 2.2 ± 2.8 |

<sup>a</sup> Included are peptides and sequence regions modified in more than one species. Modified residues are listed where known. A range of residues is listed where the exact modified residue could not be determined due to incomplete fragmentation. All peptides were in either the +3 or +4 charge states and had masses between 2200 and 3900 Da. Complete mass and charge information can be found in supplementary Table S2.

<sup>b</sup> The number of times a peptide was identified out of the total number of experiments conducted.

<sup>c</sup> Errors listed are the average and standard deviation of mass discrepancies for all peptides identified. For n = 2, the range is presented, and for n = 1, the single value for ΔM is listed.

<sup>d</sup> For Ms sGC-NT experiments, results for all constructs and ligation states (± NO, CO, etc.) are combined. In each case, the modified peptide was identified in all Ms sGC-NT constructs analyzed (Ms sGC-NT23, Ms sGC-NT13, Ms sGC-β1).

<sup>e</sup> Range of residues that were photoaffinity labeled in at least two sGC and/or H-NOX constructs.

<sup>f</sup> Specific residues identified in individual sGC and H-NOX constructs.
Mutational analysis of the proposed stimulator binding site in \textit{Ms} sGC-NT25.

CO binding constants were measured spectroscopically by monitoring the change in Soret from 433 nm (unliganded heme) to 424 nm (CO-bound complex). In the presence of 10 \( \mu \)M BAY 41-2272, CO titrations were measured with 30 nM protein in a cuvette with a 10 cm pathlength. In the absence of stimulator, CO titrations were measured with 1 \( \mu \)M protein in a cuvette with a 1 cm pathlength. Reported \( K_d^{CO} \) values are the average of two independent measurements ± range.

| Protein        | \( K_d^{CO} \) (\( \mu \)M) | \( K_d^{CO} + \text{Bay} \) (\( \mu \)M) | Ratio |
|----------------|------------------------------|---------------------------------|-------|
| Wild Type      | 26 ± 6                       | 0.4 ± 0.1                       | 65    |
| L12W           | 28 ± 10                      | 2.2 ± 0.8                       | 13    |
| T48W           | 72 ± 5                       | 1.7 ± 0.9                       | 43    |
| I66W           | 38 ± 5                       | 1.1 ± 0.1                       | 34    |
| L12W/T48W      | 49 ± 17                      | 6.7 ± 2.3                       | 7     |
Stimulator binding to the sGC \( \beta 1 \) H-NOX domain

**Fig. 1**

A. Human sGC (Full Length)

- H-NOX
- PAS
- CC
- Cyclase
- GTP
- Strep
- His\(_{6}\)

Manduca sexta sGC

- NT13
- NT23
- \( \beta 1 \)

Bacterial H-NOX Domains

- Cb SONO
- Sw, Ns, So H-NOX

B. Chemical structures

- IWP-854
- IWP-051
- BAY 41-2272
Stimulator binding to the sGC β1 H-NOX domain
Stimulator binding to the sGC β1 H-NOX domain

Fig. 3

A

| M| Y| G| F| V| N| Y| A| E| L| L| V| M| K |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
MH+ 3245.646 Da (+4)

IWP-854 (Y7), Oxidation (M1, M14)

Intensity (Counts)

B

β PAS (back)

α PAS (front)

β CC

α H-NOX

β H-NOX

β 41-42

β 365-366 361-362 (Ms)

β 374-375 370-371 (Hs)
Stimulator binding to the sGC β1 H-NOX domain

Fig 4.
Stimulator binding to the sGC β1 H-NOX domain

Fig. 5
Fig. 6

Stimulator binding to the sGC β1 H-NOX domain
Discovery of stimulator binding to a conserved pocket in the heme domain of soluble guanylyl cyclase
Jessica A Wales, Cheng-Yu Chen, Linda Breci, Andrzej Weichsel, Sylvie G Bernier, James E Sheppeck, Robert Solinga, Takashi Nakai, Paul A Renhowe, Joon Jung and William R. Montfort

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