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Insights into soluble guanylyl cyclase activation derived from improved heme-mimetics

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Abstract

Recently the structure of BAY58-2667 bound to the *Nostoc* sp. H-NOX domain was published. Based on this structural information, we designed BAY58-2667 derivatives and tested their effects on soluble guanylyl cyclase (sGC) activity. Derivative 20 activated sGC 4.8-fold more than BAY 58-2667. Co-crystallization of 20 with the *Ns* H-NOX domain revealed that the increased conformational distortion at the C-terminal region of α F helix containing 110-114 residues contributes to the higher activation triggered by 20.

Soluble guanylate cyclase (sGC) is the key “receptor” for the endogenously produced gasotransmitter nitric oxide (NO) and regulates many diverse physiological processes, including vasodilation, platelet aggregation and neurotransmission¹. sGC is a heterodimeric hemoprotein, in which heme serves as the NO sensor regulating catalytic activity². Binding of NO to the ferrous (Fe²⁺) heme moiety of sGC induces structural changes that are transmitted from the N-terminal heme binding domain of the β 1 subunit to the catalytic C-terminal domain increasing production of the second messenger cyclic guanosine-3',5'-monophosphate (cGMP) from guanosine 5'-triphosphate (GTP)^{1,3}.

Agents that stimulate sGC activity by releasing NO have been used in clinical practice for well over a century. Examples of drugs that exert their action through sGC stimulation are nitroglycerin and organic nitrites/nitrates that are used to treat angina pectoris and sodium nitroprusside that is used to manage hypertensive emergencies⁴. NO donors, however, suffer serious drawbacks. Tolerance to nitrites/nitrates develops and in many cases limits their therapeutic usefulness⁵. About a decade ago, interest in sGC was revived following the discovery of NO-independent activators and stimulators that have been proposed as promising agents for the treatment of cardiovascular and pulmonary diseases⁶⁻⁷. These agents fall into two categories: those that require the presence of heme to enhance sGC activity (termed sGC stimulators, exemplified by YC-1 and BAY 41-2272)⁸ and those that

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Supporting Information. Synthesis details, 1H-NMR and 13C-NMR of synthesized compounds, pharmacological and molecular biological methods are in supplemental information and available free of charge via the internet <http://pubs.acs.org>.

are able of activating the heme-less sGC enzyme (termed activators, exemplified by HMR-1766 and BAY 58-2667)⁹. These new sGC activators are valuable pharmacological agents, as they are able to enhance sGC activity even when the enzyme is insensitive to both endogenously produced NO and exogenously applied NO donors⁶. A prominent member of these heme-independent sGC activators is BAY 58-2667 (cinaciguat), which was tested as a candidate drug in clinical trials for acute decompensated heart failure¹⁰. This agent exhibits vasodilator and antiplatelet activity, a potent antihypertensive effect and a hemodynamic profile similar to that of nitrates⁶.

Recent crystallographic and mutagenesis studies yielded insights into the binding and activation mode of cinaciguat¹¹. The X-ray structure of a homologous heme–nitric oxide/oxygen binding (H-NOX) domain from *Nostoc* sp. with cinaciguat provides evidence that the sGC activator displaces the native heme prosthetic group from the heme pocket. Binding of cinaciguat leads to a structural change of the heme-free sGC, which is probably similar to the binding of NO to the non oxidized, heme-containing enzyme. The binding mode of cinaciguat to H-NOX can be attributed to two main critical features, the hydrophilic carboxybutyl and the hydrophobic aromatic western part. Although, both carboxylates mimic the interaction of the heme propionate side chains in the enzyme, correlation of the crystal structure data with the published structure activity relationships shows that the butyl carboxylate plays a dominating role. Whereas the benzoic acid moiety interacts only with Arg138 and the backbone nitrogen of Tyr2, the aliphatic carboxylate group provides hydrogen bonds to all three amino acids of the Y-S-R motif (Figure 1A), a conserved motif in gas-sensing domains found in both prokaryotes and eukaryotes; this motif is proven to be crucial for cinaciguat activity¹².

BAY 58-2667 (cinaciguat) features a long hydrophobic region, which folds up in the heme cavity. The phenylethylamino group interacts with Leu101, while the 4-phenylethylbenzol is flanked by Tyr83 and the benzoic acid carboxylate moiety of the ligand itself¹¹. On the basis of this structural information, our goal was to design derivatives of BAY58-2667 to optimize the interaction of the ligand with the binding domain by variation of the chain lengths at the critical positions (Figure 1B) and to test the effects of these derivatives on sGC activity to gain structure-function insights relating to sGC activation. In the case of the butyl carboxylate moiety, we decided to both extend and shorten the chain by methylene groups in order to investigate its optimal length. On the other hand, the X-ray crystal structure reveals considerable flexibility in the terminal phenyl moiety. The partial displacement of Phe112 suggests that even a longer chain could be tolerated. Hence, we hypothesized that elongation of the hydrophobic tail of cinaciguat by incorporation of various phenyl- or aryl ether groups would give the required length for aromatic interaction of its terminal phenyl ring with Phe112.

Results and Discussion

Chemistry

The target compounds were synthesized following two different strategies, each of which yielded intermediates that allow for diversification in the last step of the synthetic routes. The alkyl acid analogues of cinaciguat were obtained as shown in Scheme 1. The starting material 4-(phenethylphenyl)methanol **1** was synthesized according to known procedures¹³⁻¹⁷ and transformed to the corresponding bromide by means of Appel halogenation¹⁸. Etherification with methyl 2-(2-hydroxyphenyl)acetate¹⁹⁻²⁰ in the presence of K₂CO₃ in acetonitrile yielded methylester **2**, which was hydrolyzed, followed by treatment with methyl 4-(aminomethyl)benzoate hydrochloride²¹⁻²² under standard conditions. The resulting secondary amide **3** was then reduced with borane-tetrahydrofuran complex to the corresponding amine, which was coupled with the appropriate acyl chlorides

34-36 to afford the tertiary amides 4-6. A second reduction with borane-tetrahydrofuran complex furnished tertiary amines 7-9. Hydrolysis of the methylester groups with NaOH in dioxane yielded the hydrochloride salts 10 and 11, as well as their parent compound cinaciguat²³.

The synthesis of the derivatives with an elongated aromatic hydrophobic tail included generally the same key transformations (Scheme 2). Methylester **12**¹⁹⁻²⁰ was alkylated using commercially available 1-bromo-4-(bromomethyl)benzene under the conditions of the Williamson ether synthesis. Subsequent hydrolysis of the ester moiety with LiOH gave the corresponding acid which was transformed to amide **13** by condensation with methyl 4-(aminomethyl)benzoate hydrochloride. Reduction of the amide functionality with borane-tetrahydrofuran complex and acylation of the resulting secondary amine **14**²³ with methyl 5-chloro-5-oxopentanoate led to the corresponding tertiary amide **15**. After a second reduction with borane-tetrahydrofuran complex the tertiary amine **16** was obtained, which represents a general intermediate for the following diversification by Suzuki-Miyaura reaction. Coupling of derivative **16** with commercially available boronic acids **17-19** led to the corresponding products, which after saponification afforded the target compounds **20-22** as hydrochloride salts.

As in both approaches the tertiary amides **4-6** (Scheme 1) and **15** (Scheme 2) were obtained as intermediates, we decided to synthesize the corresponding derivatives having free carboxy groups. Consequently, direct hydrolysis and precipitation of amides **4-6** furnished the hydrochloride salts **23-25**, while tertiary amide **15** was first coupled with boronic acids **17** and **18** in a Suzuki-Miyaura reaction before saponification to give **26** and **27** (Scheme 3).

It should be noticed that the NMR spectra of the tertiary amides (**4-7**, **15**, and **23-27**) showed a 2:1 ratio of rotamers. Furthermore we observed general signal broadening in the ¹H-NMR of the final tertiary amines occurred due to hindered rotation around the amide bonds (**11**, **20**, **21**, **26** and **27**).

Pharmacology

Synthesized analogues were initially tested for their ability to stimulate cGMP accumulation in cultured aortic smooth muscle cells. Shortening and elongation of the carboxy alkyl moiety length by one carbon atom (compound **10** and **11**) reduced its ability to stimulate cGMP accumulation. Similar results were obtained during optimization of the primary HTS hit BAY W1449 that lead to identification of BAY 58-2667⁹. This indicates that the chain length of five carbon atoms was optimal. Introduction of an amide group inhibited cGMP production by approximately 80% (compounds **23-25**; Suppl. Table S1), indicating the importance of a tertiary amine moiety for activity. Replacement of the phenylethyl group of cinaciguat by a p-methoxybenzyl or benzyl group reduced markedly the activity of the derivatives **22** and **27**. On the other hand introduction of an aryl ether moiety resulted in compound **20** with significantly enhanced ability for raising cGMP levels. In contrast, introduction of an amide group in the carboxy-alkyl moiety lead to derivative **26** (Table S1) that exhibited a markedly reduced activity. Similarly, replacement of the phenyl-ether group of **20** by a benzyl ether (compound **21**), lead to a significant reduction in cGMP accumulation. In general the amide derivatives were far less potent than the amine derivatives (**27**, Table S1).

Compound **20** enhanced cGMP formation in a concentration-dependent manner, while BAY 58-2667 reduced **20**-induced cGMP accumulation when used in excess, behaving as a partial agonist (Figure 2). However, when the two compounds were used at equimolar

concentrations BAY 58-2667 did not affect the response to **20**, suggesting that **20** has stronger affinity for the sGC heme pocket than BAY 58-2667.

Structure of **20** bound C139A *Ns* H-NOX

The mutant C139A H-NOX protein was obtained after an extensive effort to generate surface mutant variants of the 1-182 *Ns* H-NOX protein to yield a variant with improved crystallizability, reproducibility, and diffraction. As a result, we utilized the C139A variant for co-crystallization studies of BAY58-2667 analogs including the **20** compound, whereas previously the wild type *Ns* H-NOX protein was used for the BAY58-2667 studies¹¹. The C139A mutation itself causes very limited structural changes likely due to this being a solvent-exposed residue and being distant from the heme pocket²⁴. For the **20**-C139A complex, heme density was absent, yet strong electron density was present, after initial refinement, for the two copies of **20**, one in each of the monomers (Figure 3A and 3B). Compound **20** makes the following interactions. The carboxyl-butyl moiety of **20** makes hydrogen bond and/or salt-bridge interactions with the YxSxR motif involving side chains of Y134, S136, and R138. The benzoic acid carboxylate moiety of **20** makes a salt-bridge with R138 and an hydrogen bond with the main chain nitrogen of Y2 (Figure 3A). Electron density for the **20**'s hydrophobic moieties is well defined except for the terminal phenyl ring (Figure 3B). These hydrophobic moieties of **20** make extensive hydrophobic interactions with many hydrophobic residues: L4, W74, T78, Y83, F97, L101, L104, V108, L148, and L152. The loop region encompassing residues 109-113 is less well defined resulting in increased temperature factors, a small break in density for this region near 111-112, and lack of density for the side chain of F112.

Comparison of BAY58-2667-*Ns* H-NOX and **20**-C139A structures

20 is a derivative of BAY58-2667 and, when bound, activates soluble guanylyl cyclase 4.8-fold higher than BAY58-2667. To probe the structural basis for this intriguing activation enhancement, the structures of **20** bound C139A *Ns* H-NOX and BAY58-2667 bound *Ns* H-NOX structures were compared. Both of these compounds are trifurcated and contain two identical carboxylate moieties while the third moiety extending from the tertiary amine is somewhat different, particularly at its terminal end containing benzyl rings. Therefore, the two compounds make very similar interactions within the heme pocket. While comparing both protein structures, we observed that the important α F helix does not change its position, whereas the loop region comprising of residues 110-114 at the carboxyl terminus of α F helix does have an altered conformation in the **20** structure (Figure 4A). Electron density for this region in the **20**-C139A structure is weak and not well-defined, particularly for residues S111 and F112. Structural analysis reveals that the additional benzyl ring of the **20**, not ordered either, likely sterically displaces the phenyl ring of the F112 side chain and pushes this loop region further away from the heme pocket (Figure 4B). These changes cause the following shifts: the side chain of F112 is pushed 7.7 Å away, the carbonyl bond of S111 by 2.6 Å, and the P113 ring by 2.5 Å (Figure 4C). These changes are observed in both monomers in the crystal structure. As previously hypothesized¹¹, this loop region is likely critical for transmitting the activation signal to the rest of sGC upon NO or BAY58-2667 activation. While BAY58-2667 binding to H-NOX also shifts this region relative to heme-bound H-NOX, **20** binding causes the additional effect of displacing the side chain of F112 and the adjacent loop residues due to the added terminal benzyl ring moiety (Figure 4B and 4C). Therefore, we hypothesize that this additional conformational distortion of the H-NOX 110-114 loop region may contribute towards the higher activation of sGC by **20** in comparison to that of BAY58-2667. We previously hypothesized that this H-NOX region could perhaps directly interact with the catalytic domains of sGC¹¹, as the Marletta lab had initially shown that the isolated H-NOX domain interacts and inhibits a catalytic domain construct²⁵. Increased distortion of this region by **20** pushing F112 towards the exterior

could lead to increased alteration of the interaction between the H-NOX and the catalytic domain thereby possibly explaining the increased activation of sGC by **20**. Residue 112 in sGC is a tyrosine; in order to determine the effect of Y112 on the activation of sGC induced by **20**, we mutated this residue to alanine or introduced a glycine in position 112 followed by the tyrosine that moved to position 113. Both the Y112A and G112 mutants showed significantly reduced NO-induced cGMP accumulation, suggesting that disrupting this region of sGC either causes loss of heme or disrupts the signal transmission triggered by heme distortion following NO binding (Figure S1). The Y112A mutant responded to **20** by producing less cGMP, while insertion of a glycine in position 112, that probably causes a more drastic structural change, reduced responses to compound **20** by half.

Conclusions

Using a structure-guided design approach, we designed and synthesized a BAY 58-2667 analogue that contains an additional aryl ether moiety (**20**, also termed MaW-4). This modification yielded a significant enhancement of sGC activation compared to BAY 58-2667. Based on structural data, we predicted that **20** (MaW-4) enhances sGC activity by acting on Y112 and pushing this residue outwards. Indeed, mutating Y112 to A or inserting a G before the Y reduced the ability of **20** to increase cGMP synthesis, providing further evidence that the carboxy terminal region of α F helix participates in transmitting the conformational change from the N- to the C-terminus of sGC in response to sGC activators.

Experimental Section

General procedures

The syntheses of selected compound **20** from the intermediate **16** is described below as representative. Briefly, to the bromide **16** solution in DMF, Na₂CO₃ (5 eq.) and Pd(PPh₃)₂Cl₂ (3.5 mol%), boronic acid **17** (1.2 eq.) was added. The resulting mixture was heated under reflux for 40 min and was then diluted with EtOAc. The organic phase was washed with 5 % NaHPO₃ solution, water and brine, and dried with Na₂SO₄. The coupled product was purified via column chromatography (SiO₂, n-hexane/EtOAc) after all volatiles were removed. The product was dissolved in dioxane (3 mL/0.5 mmol) and NaOH solution (40 wt%, 0.3 mL/0.5 mmol) was added. The mixture was heated for 18 h at 60°C. After all volatiles were removed, the residue was dissolved in water and acidified with HCl (1 M, pH 4). The white precipitate was filtered and dried under vacuum. The purity of all tested compounds was analyzed using AQUITY Ultra Performance liquid chromatography (UPLC) system coupled to a Photodiode Array (PDA) Detector (Waters Corporation, Milford, MA, USA) and was found to be 95%. Detailed synthesis information and spectra of all compounds can be found in the Supporting Information.

Crystallization and structure determination

The colorless **20**-C139A *Ns* H-NOX protein complex were crystallized using sitting drop crystallization method at 22°C with the protein concentration of ~12 mg/ml for each set-up. Crystallization condition for the **20**-C139A *Ns* H-NOX complex was 1.9 M sodium malonate, pH 7.0. Crystals were cryo-protected in 3.0 M sodium malonate, pH 7.0 prior to dunking the crystals in liquid nitrogen for storage and data collection. For data collection see supplemental information. The structure is deposited in the Protein Data Bank with the PDBid 4JQH.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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ABBREVIATIONS

sGC	soluble guanylyl cyclase
H-NOX	heme–nitric oxide/oxygen binding

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Figure 1. Schematic representation of the binding mode of BAY 58-2667 (cinaciguat) observed in the crystal structure with H-NOX domain (A). Summary of strategy to design of cinaciguat analogues (B).

Figure 2.

Competition between **20** and BAY 58-2667. Confluent RASMCs were treated with vehicle, compound **20** (0.01-1 μ M) in the presence or absence of BAY 58-2667 (1 μ M) for 15min. cGMP was extracted and measured as described in Methods section. Data are presented as means $\pm$ SEM; n=4; *P<0.05 from respective **20**.

Figure 3.

(A) Omit electron density maps for **20** (red sticks) bound to heme binding pocket of C139A H-NOX (gray sticks, interacting amino acids are labeled) (2Fo-Fc omit density in blue at 0.9σ and FoFc omit density in green @ 3.0σ). (B) 2Fo-Fc map (blue color at 1.0σ) for **20** (in red color) showing no well defined density for the terminal benzyl ring. For comparison, BAY 58-2667 is shown in yellow and the residues are labeled.

Figure 4.

(A) Superposition of **20** bound C139A structure and BAY 58-2667 bound *Ns* H-NOX structure, **20** is shown in red spheres and BAY 58-2667 is shown in yellow spheres. C139A: **20** protein is shown in gray cartoon and sticks while *Ns* H-NOX: BAY 58-2667 protein is shown in yellow cartoon and sticks. (B) **20** is shown in red sticks and BAY 58-2667 is shown in yellow sticks. C139A protein is shown in gray sticks and *Ns* H-NOX is shown in yellow sticks. 2Fo-Fc map is shown in blue at 1 σ . The terminal benzyl ring of **20** when bound to *Ns* H-NOX clearly forces the phenyl ring of F112 away from the loop. (C) Distances between the atoms of the superposed loop region comprising of 111-113 residues.

Scheme 1. Synthesis of cinaciguat and its derivatives

Reagents and conditions: (a) CBr_4 , PPh_3 , DCM, rt, 2h, 98%; (b) methyl 2-(2-hydroxyphenyl)acetate, K_2CO_3 , MeCN, reflux, 3.5h, 90%; (c) $\text{LiOH}\cdot\text{H}_2\text{O}$, THF/ H_2O (4:1), rt, 16h, quant; (d) methyl 4-(aminomethyl)benzoate hydrochloride, CDI, DMAP, DCM, rt, 3h, 98%; (e), $\text{BH}_3\cdot\text{THF}$, THF, reflux, 3h, then 10% HCl in MeOH, reflux, 1h, 85%; (f) acylchloride 34-36, pyridine, DMF, rt, 1h (43-97%); (g) $\text{BH}_3\cdot\text{THF}$, THF, rt, 18h, then 10% HCl in MeOH, reflux, 1h, rt, 22-97%; (g), 40% NaOH, dioxane, 60°C , 18h, then H_2O , HCl (pH 4), rt, 59-92%.

Scheme 2. Synthesis of biphenylic analogues of cinaciguat

Reagents and conditions: (a) 1-bromo-4-(bromomethyl)benzene, MeCN, K_2CO_3 , reflux, 4h, 87%; (b) $LiOH \cdot H_2O$, THF/ H_2O (4:1), rt, 16h, quant.; (c) $SOCl_2$, DMF, DCM, reflux, 2h, then methyl 4-(aminomethyl)benzoate hydrochloride, DCM, NEt_3 , 0°C to rt, 16h, 83%; (d) $BH_3 \cdot THF$, THF, reflux, 3h, then 10% HCl in MeOH, reflux, 1h, 69%; (e) methyl 5-chloro-5-oxopentanoate, pyridine, DMF, rt, 1h, 84%; (f) $BH_3 \cdot THF$, THF, rt, 18h, then 10% HCl in MeOH, reflux, 1h, 58%, (g) boronic acid 17-19, $Pd(PPh_3)_2Cl_2$, DMF, $Na_2CO_3(aq)$, reflux, 40 min, 69-78%; (h) 40% NaOH, dioxane, 60°C, 18h then H_2O , HCl (pH 4), rt, 86-89%.

Scheme 3. Synthesis of the amide derivatives of cinaciguat

Reagents and conditions: (a) 40% NaOH, dioxane, 60°C, 18h then H₂O, HCl (pH 4), rt, 71-92%; (b) boronic acid 17-19, Pd(PPh₃)₂Cl₂, DMF, Na₂CO₃(aq), reflux, 40 min, 33-55%.