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Drug Discovery

The Chemistry and Biology of Soluble Guanylate Cyclase Stimulators and Activators

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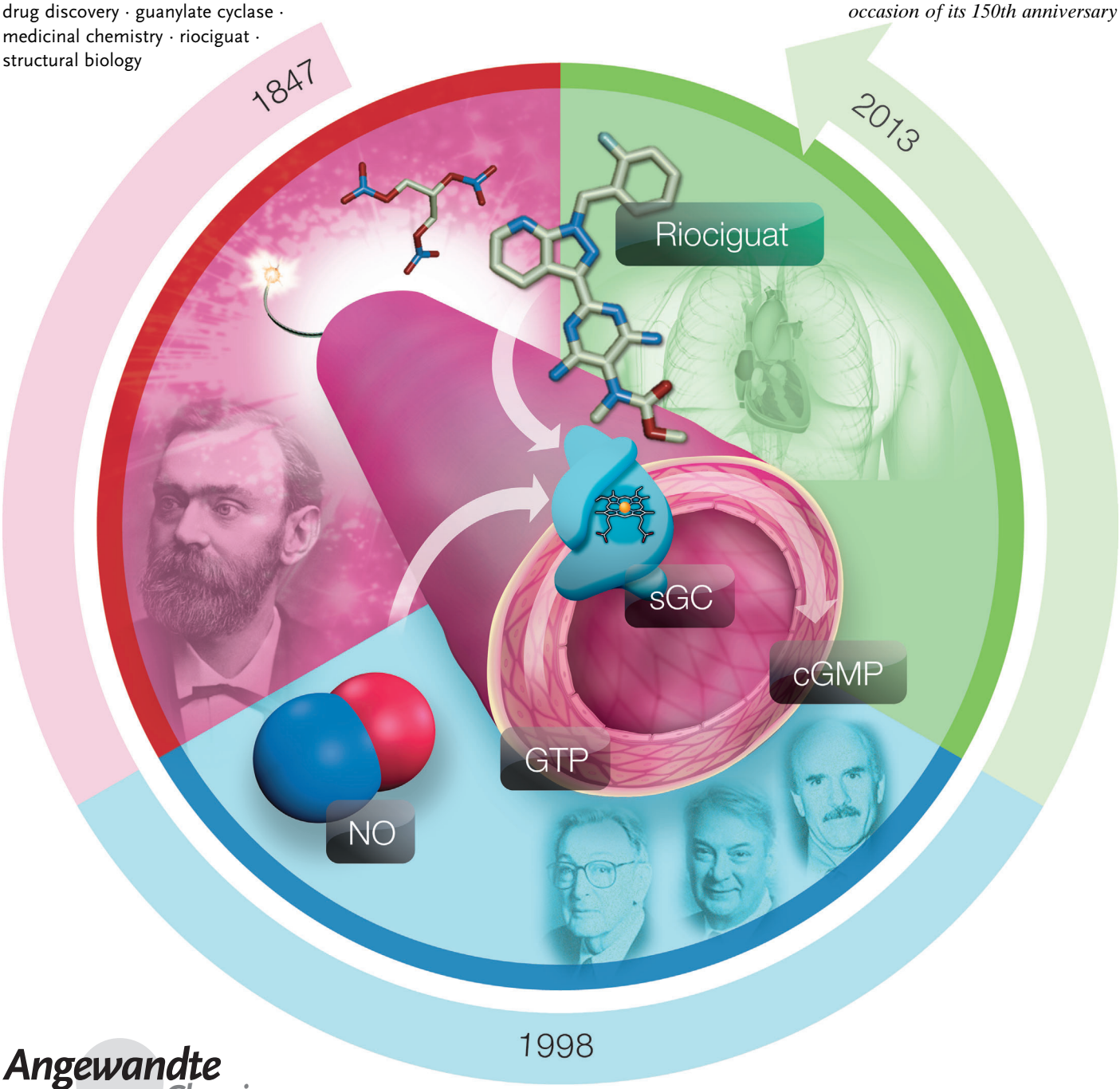
Dedicated to the Bayer company on the occasion of its 150th anniversary



The vasodilatory properties of nitric oxide (NO) have been utilized in pharmacotherapy for more than 130 years. Still today, NO-donor drugs are important in the management of cardiovascular diseases. However, inhaled NO or drugs releasing NO and organic nitrates are associated with noteworthy therapeutic shortcomings, including resistance to NO in some disease states, the development of tolerance during long-term treatment, and nonspecific effects, such as post-translational modification of proteins. The beneficial actions of NO are mediated by stimulation of soluble guanylate cyclase (sGC), a heme-containing enzyme which produces the intracellular signaling molecule cyclic guanosine monophosphate (cGMP). Recently, two classes of compounds have been discovered that amplify the function of sGC in a NO-independent manner, the so-called sGC stimulators and sGC activators. The most advanced drug, the sGC stimulator riociguat, has successfully undergone Phase III clinical trials for different forms of pulmonary hypertension.

From the Contents

1. Introduction

In retrospect, the discovery of many drugs was anything but planned.[1–3] Researchers often refer to serendipity when they encounter a breakthrough innovation in one field when searching for something completely different. The discovery of nitroglycerine is a prime example of such a case. It was discovered in 1847 by the Italian chemist Ascanio Sobrero (1812–1888) at the University of Turin; however, Sobrero already noted that exposure to this chemical caused headaches (we know today that this is caused by cerebral vasodilation). The Swedish inventor Alfred Nobel (1833–1896) studied nitroglycerine and found that embedding the chemical in diatomaceous earth makes it less sensitive to impact, more easily controllable, and therefore more transportable. He called the formulation Dynamite and patented it in 1867.[4–6] When produced on an industrial scale, co-workers at his production sites complained about unpleasant effects of the substance, as it caused headaches and dizziness. Physicians at that time started experimenting with organic nitrates. In 1867 Sir Lauder Brunton (1844–1916), a Scottish physician, found that organic nitrates were effective in relieving the chest pain of angina pectoris.[7] In his famous publication in *The Lancet* in 1879 entitled "Nitroglycerine as remedy for angina pectoris",[8] the British physician William Murrell described his results concerning self-experiments and treatment of patients with nitroglycerine.[9]

Ironically, Nobel himself at the end of his life suffered from heart disease and angina pectoris. Less than two months before his death, Alfred Nobel wrote a note to a colleague: "Isn't it the irony of fate that I have been prescribed nitroglycerine to be taken internally! They call it Trinitrin, so as not to scare the chemist and the public." He declined to take it for his angina pectoris.[10]

In contrast to Nobel's own reluctance, nitrodilators such as nitroglycerine have been broadly applied since that time for treating angina attacks and heart failure and are still used today, despite unpleasant side effects and many limitations.[11,12]

But what was the mechanism by which this powerful explosive chemical, discovered in the 19th century, relieves angina pectoris chest pains and dilates blood vessels? The answer to this question was not to come for another century.

In 1977 Ferid Murad discovered that nitrates only work physiologically when they have been broken down to nitric oxide (NO).[13,14] The gas, which is also categorized as an environmental pollutant, relaxes smooth muscle cells. In 1978 Robert Furchgott (1916–2009) found that a substance in the endothelium (internal wall of the vessel) relaxes blood vessels, calling it endothelium-derived relaxing factor (EDRF).[15–17] The exact nature of EDRF was up to this point unknown. Interestingly, Louis Ignarro independently came to similar conclusions around the same time,[18–26] and both researchers presented evidence at conferences during 1986 demonstrating that EDRF is in fact NO. In 1998 the Nobel Prize in physiology or medicine was awarded jointly to Murad, Ignarro, and Furchgott for their discovery of the mechanism of action of NO in the body.[14,26] Notably, they received the most prestigious prize in science for proving that the substance Nobel developed as an explosive acts physiologically by releasing NO.

In more detail, NO acts via soluble guanylate cyclase (sGC), an enzyme with high affinity for NO.[27] sGC is an important regulator in the cardiovascular system and present in smooth muscle cells of blood vessels and in platelets.[28–31] NO is synthesized by various nitric oxide synthases from L-arginine, including endothelial nitric oxide synthase (eNOS), neuronal nitric oxide synthase (nNOS), and the inducible isoform (iNOS). Within endothelial cells, the inner layer of cells in arteries, NO is produced by endothelial nitric oxide synthase (eNOS) and diffuses rapidly to deeper-lying smooth muscle cells, where it stimulates sGC (Figure 1). This results

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in an intracellular increase of the messenger substance cyclic guanosine-3′,5′-monophosphate (cGMP), triggering a variety of physiological responses, including dilation of blood vessels (thereby lowering the blood pressure) and an improved circulation. The pathogenesis of various diseases, and particularly of the cardiovascular system, has been linked to an inappropriate stimulation of sGC.[32–38]

sGC itself is a cytosolic, heterodimeric protein consisting of an α- and a β-subunit with a prosthetic heme group located in the β-subunit (heme-binding domain).[39–45] NO stimulates sGC activity by binding to the Fe^{2+} of the heme group, which induces cleavage of a Fe^{2+}–histidine (His^{105}) bond, leading to conformational reorganizations that are propagated into the catalytic subunit, where cGMP production is in turn increased. However, this NO-induced switch mechanism is an oversimplification of a more complex process, as will be discussed in Section 4 in more detail. cGMP regulates various effector systems, such as cGMP-dependent protein kinases, ion channels, and phosphodiesterases. Further downstream, these effector systems mediate various physiological and tissue-protective effects, including vasorelaxation and inhibition of smooth muscle proliferation, leukocyte recruitment, and platelet aggregation. This axis is referred to as the NO-sGC-cGMP pathway (Figure 1).[46,47]

sGC can exist in two different forms (Figure 2): The native heme-containing or reduced form of sGC, which is the endogenous receptor for NO, and the heme-free form of sGC. Under conditions of oxidative stress, which is thought to be

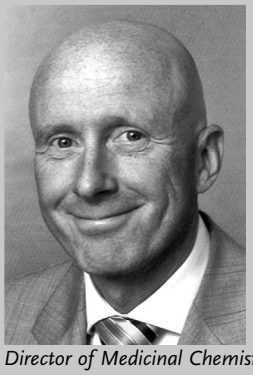
Markus Follmann studied chemistry in Germany and Japan. In 2000 he finished his doctorate with Prof. H. Kunz in Mainz. After a two-year postdoctoral training with Prof. K. C. Nicolaou in La Jolla he joined the pharma research of Aventis AG in Frankfurt in 2003. In 2008 he moved to Wuppertal to join Bayer Healthcare, where he served as a project leader for several projects in cardiovascular indications. Since 2012 he has been Director of Medicinal Chemistry. He is co-author of more than 60 publications and patent applications.

Ingo V. Hartung studied chemistry in Hannover (Germany) and at Stanford University (USA), followed by a Ph.D. thesis with Prof. H. M. R. Hoffmann at the University of Hannover. After a postdoctoral stay with Prof. Chaitan Khosla (Stanford University), he joined Schering AG in 2004. Over the last 9 years he has contributed to the optimization of lead compounds for cancer as well as cardiological projects, from 2009 to 2011 as a Labhead with Bayer HealthCare in Wuppertal/Germany. Since 2011 he has been in charge of a fermentation and cell culture lab within Bayer HealthCare in Berlin. His current research focus is on the preparative scale biotransformation of drugs into their metabolites and new trends in biocatalysis.

Nils Griebenow studied chemistry in Marburg and at the Max-Planck-Institute for Coal Research in Mülheim an der Ruhr, where he carried out his doctoral work under the supervision of Prof. Manfred T. Reetz. In 1995, he joined the Bayer AG setting up combinatorial chemistry at the central research facility. In 2002 he moved to the pharma division of Bayer, working as a medicinal chemist on the lead identification and optimization in various therapeutic areas, such as CNS disorders, atherosclerosis, cardiovascular diseases and cancer. He is co-inventor of more than 40 patents and patent applications, 19 of which are related to soluble guanylate cyclase stimulators and activators.

Franz-Josef Mais studied chemistry in Mainz und Düsseldorf and wrote his Ph.D. thesis with Prof. H.-D. Martin. He joined Bayer in 1985 in the business unit Organica working on process development for basic and fine chemicals. In 2002 he moved to HealthCare and since then he has been active in process development mainly for cardiological and oncological drugs. He is the developer for commercial synthesis of the sGC-stimulator riociguat.

Michael G. Hahn studied chemistry at the University of Wuppertal and he obtained his doctorate with Prof. H.-J. Altenbach in 1998. After postdoctoral research at the University of Cambridge (UK) in the group of Prof. S. V. Ley, he moved to the institute for Medicinal Chemistry of Bayer HealthCare and has worked there since then in the area of sGC Activators.



Joachim Mittendorf studied chemistry in Goettingen and received his doctorate with U. Schöllkopf in 1989. After postdoctoral research with K. P. C. Vollhardt at UC Berkeley he joined the pharma research division of Bayer AG in 1990 as a medicinal chemist working on antiinfectives. During a job rotation from 1994–1996 he worked on the scale-up of drug candidates in chemical development. He was then project leader in Medicinal Chemistry on different projects in the indications stroke, asthma, and cardiovascular disease. Since 1999 he has been Director of Medicinal Chemistry at Bayer HealthCare in Wuppertal. Since 2002 he has also been responsible for an integrated research IT platform. In 2009 he was appointed "Honorarprofessor" for Medicinal Chemistry at the Univ. of Leipzig.

involved in the development of many cardiovascular diseases, reactive oxygen species (ROS) are formed.[49] ROS are able to oxidize the heme iron of sGC ($Fe^{2+} \rightarrow Fe^{3+}$), resulting finally in a loss of the heme group.[50] The heme-free form of sGC is no longer responsive to NO and thus termed dysfunctional. Furthermore, the heme-free sGC is rapidly degraded by ubiquitinylation.[50–52]

During the last 15 years, two compound classes have been discovered that are capable of activating both forms of sGC in a NO-independent manner:[27,47] The heme-dependent sGC stimulators and the heme-independent sGC activators. sGC stimulators share a dual mode of action: they synergize with endogenous NO, and furthermore they are also capable of directly stimulating the native form of the enzyme independent of NO. In contrast, sGC activators are able to activate the pathologically changed heme-free sGC; in other words, when it is in a condition in which the body's own NO hardly has any effect.

Both stimulation of the native or activation of the heme-free sGC lead to an increased cGMP formation.[27] The chemistry and biology of these two novel classes of drugs, sGC stimulators and sGC activators, some of which currently being investigated in clinical trials, will be further discussed in this Review.

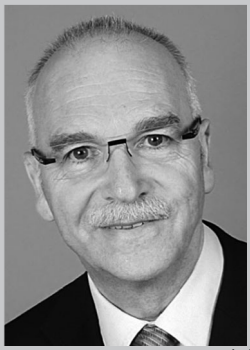
2. sGC Stimulators

2.1. Discovery of NO-Independent, Heme-Dependent sGC Stimulators

In 1994, scientists at Bayer started a screening for substances that could induce an increase in NO synthesis and thereby stimulate sGC in porcine endothelial cells.[27,47] These studies involved measurement of cGMP levels by radioimmunoassay, leading to the unexpected discovery of NO-independent sGC stimulators. At the same time, researchers at the National Taiwan University Taipei and Yung Shin Pharmaceuticals, Taiwan reported that a benzyl indazole compound named YC-1 (**1**; Scheme 1)[53] inhibited platelet aggregation by stimulation of cGMP synthesis.[54] YC-

Martina Schäfer studied chemistry and German literature in Göttingen and did her Ph.D.in crystallography with Prof. G. M. Sheldrick. In 1999 she joined the research team of the former Schering AG in Berlin as a protein crystallographer. After six years she moved to Wuppertal to reinforce the crystallography group of the Bayer Schering Pharma AG; since 2012 she has been in Berlin as a Senior Scientist at Bayer HealthCare. The focus of her research is now the 3D structure determination of proteins in complex with their new lead structures relevant for cardiologic indications.

Hartmut Schirok studied chemistry in Göttingen and completed his Ph.D. in 1999 with Prof. L. F. Tietze. After a postdoctoral stay in Prof. B. M. Trost's group in Stanford he joined the pharma research of Bayer in 2000. As Senior Research Scientist he works in cancer, heart diseases, and ophthalmology projects.



Johannes-Peter Stasch is a Chief Scientist in Cardiovascular Research at Bayer Pharma in Wuppertal, and Honorary Professor of Drug Discovery at the University Halle-Wittenberg. He studied chemistry and pharmaceutical sciences, earned a doctorate from the University of Würzburg, and received his Habilitation for Pharmacology at the University Halle-Wittenberg. He has more than 30 years' experience in the areas of biochemistry, pharmacology, and drug discovery. He has played the leading role in the discovery and development of sGC stimulators and sGC activators. His research has led to more than 140 original publications, 25 book chapters, 190 patent applications, several development compounds, and finally riociguat. More recently he has been elected as a member of the National Academy of Sciences, Leopoldina..

Friederike Stoll obtained a degree in Pharmacy in Berlin. She then joined the group of Prof. H.-D. Höltje in Düsseldorf and completed her PhD in 2001. After a Postdoc and two years with Novartis she joined the Computational Chemistry department of Bayer Healthcare in 2004 and worked predominantly on cardiovascular projects. Since 2011 she has been working in the R&D information department of Bayer HealthCare.

Alexander Straub studied chemistry in Stuttgart, obtained his doctorate with Prof. F. Effenberger, and then joined the pharmaceutical research division of Bayer AG as a medicinal chemist. After ten years he moved to Bayer CropScience where he worked on chemical production processes in the department for process research and process development for eight years. Since 2008 he has been working as Principal Research Scientist on cardiovascular indications at Bayer HealthCare. He is co-author of more than 50 publications and 60 patent applications and a co-inventor of the sGC-stimulator riociguat.

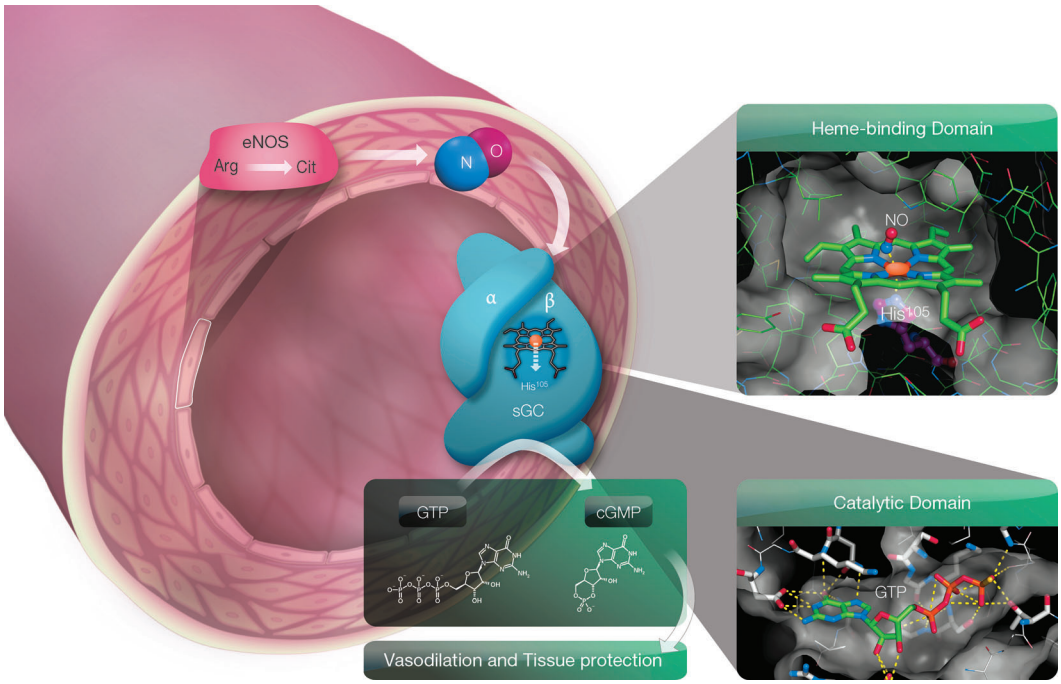


Figure 1. The NO-sGC-cGMP signal transduction pathway and crystal structure analogues of the heme-binding and catalytic domain of the human soluble guanylate cyclase (sGC). The structures depicted are based on the solved crystal structures of the heme-binding protein of cyanobacterium *Nostoc sp.* (pdb entry 2O0C)[130] with sequence homology of 35% to the sGC heme-binding domain, and the mammalian AC5 catalytic domain (pdb entry 3C14).[48]

1 (**1**) was subsequently characterized as a direct NO-independent, but heme-dependent, sGC stimulator. It stimulated isolated sGC by a factor of 30 to 40 at 100 μM, and showed a strong synergistic effect when combined with NO-releasing compounds and a loss of stimulation after oxidation or removal of the prosthetic heme moiety of sGC.[54–57]

YC-1 (**1**) exhibited a promising profile in various pharmacological studies. However, along with its relatively weak sGC stimulating potency, it revealed a poor pharmacokinetic profile and a lack of specificity as it was found to inhibit phosphodiesterases and to modulate many cGMP-independent effects.[47] Therefore, further optimization of potency, pharmacokinetic properties and specificity was required to realize the full therapeutic potential of this novel class of drugs.

2.2. First Advances towards Highly Specific and Potent sGC Stimulators

Based on these initial results, extensive structure–activity relationship (SAR) studies were performed at Bayer to systematically optimize the structure of YC-1. The in vitro potency of the compounds was assessed by two different methods, a cGMP formation assay in sGC overexpressing Chinese hamster ovary (CHO) cells and a functional assay based on the inhibition of phenylephrine-induced contraction of rabbit aortic rings.[58] A first breakthrough in terms of improved potency resulted from the replacement of the benzyl indazole moiety of YC-1 by a (2-fluorobenzyl)pyrazolopyridine moiety and, even more importantly, the exchange of the (hydroxymethyl)furan portion for a 5-substituted 4-aminopyrimidine or 4,6-diaminopyrimidine group.[59] Small-molecule X-ray structures revealed a coplanar arrangement of this biaryl system, which is apparently important for achieving high potency. The 5-cyclopropyl-4-aminopyrimidine derivative BAY 41-2272 (**2**; Scheme 1) showed a greatly improved sGC stimulating potency, with an IC_{50} of 0.3 μM for the contraction of rabbit aortic rings (YC-1, $IC_{50} = 10$ μM) and a minimum effective concentration (MEC) of 0.03 μM for cGMP formation in CHO cells (YC-1, MEC = 10 μM). In contrast to YC-1, BAY 41-2272 is a highly specific sGC stimulator, and no relevant inhibition of phosphodiesterases was observed.[27,35] Whereas the 1-(2-fluorobenzyl)-1*H*-pyrazolo[3,4-*b*]pyridine part of this new lead series turned out to be essential for potent sGC stimulating activity, the pyrimidine moiety allowed for broad variations. Further studies led to the 4,6-diamino-5-morpholino analogue BAY 41-8543 (**3**), displaying threefold higher potency in the phenylephrine-induced contraction of rabbit aorta ($IC_{50} = 0.10$ μM).

BAY 41-2272 and BAY 41-8543, however, displayed low metabolic stability and low oral bioavailability in rats, and BAY 41-2272 showed a strong inhibition as well as induction of metabolizing cytochrome P450 (CYP) enzymes.[60] Inhibition or induction of CYP enzymes by a drug bears the risk of changing the exposure of a second, coadministered drug. While these properties precluded further development, both compounds were used to study this novel class of drugs in numerous pharmacological experiments, resulting in more than 150 publications from various research groups around the world.

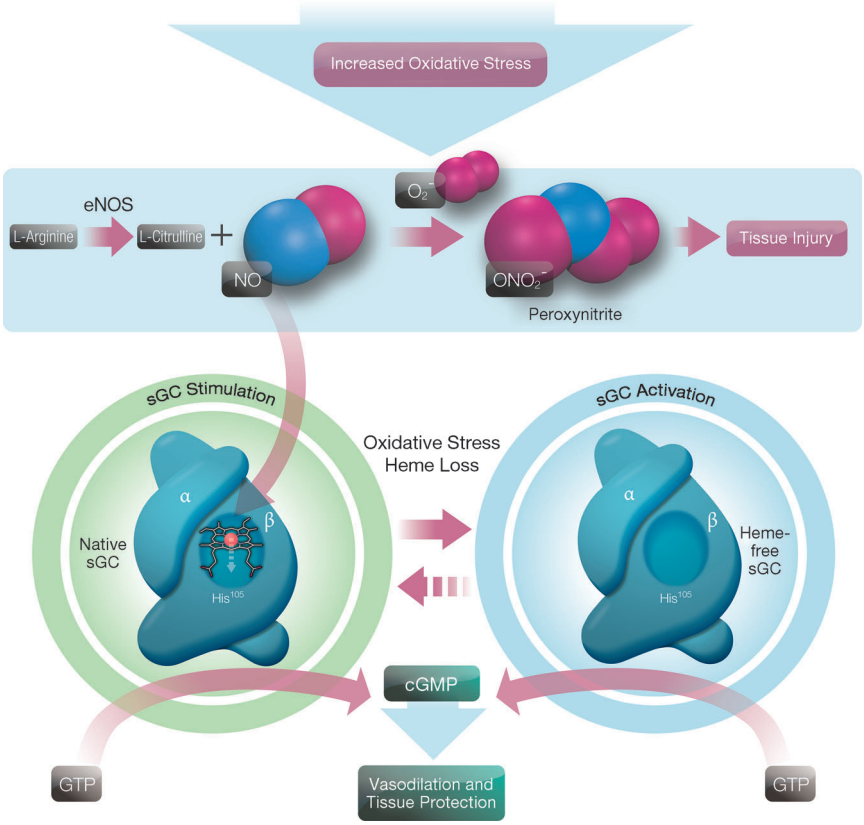


Figure 2. Soluble guanylate cyclase stimulators and activators target two different states of sGC: the NO-sensitive reduced, native sGC, and the NO-insensitive oxidized, heme-free sGC. Stimulators of sGC stabilize the nitrosyl–heme complex of the reduced sGC (shown left) and exhibit a strong synergism with NO. Activators of sGC bind to the unoccupied heme-binding complex (shown right) or displace the prosthetic heme of sGC and protect sGC from proteasomal degradation.[47]

Another optimization program based on YC-1 as a lead structure, conducted by Selwood et al. from University College London, led to the identification of CFM-1571 (**5**; Scheme 2).[61] This compound was characterized as a weak but specific stimulator of sGC (stimulation of purified sGC, $EC_{50} = 5.5\ \mu M$; platelet aggregation, $IC_{50} = 2.8\ \mu M$). However, only low oral bioavailability in rats was observed (12%), and no further progress with this series has been reported.

A novel class of sGC stimulators based on *ortho*-sulfanyl-substituted cinnamyl amides was disclosed in 2003 by scientists from Abbott.[62] A-778935 (**6**; Scheme 2) and other analogues are not structurally related to YC-1; however, it could be demonstrated that they stimulate sGC by a similar mode of action, independently of and in synergy with NO, and requiring the presence of a reduced heme moiety. A-778935 stimulated the isolated sGC NO-independently with about 20-fold higher potency than YC-1.[63] As no further progress on this compound class has been reported since 2006, it is assumed that the program has been discontinued.

2.3. Discovery and Development of Riociguat

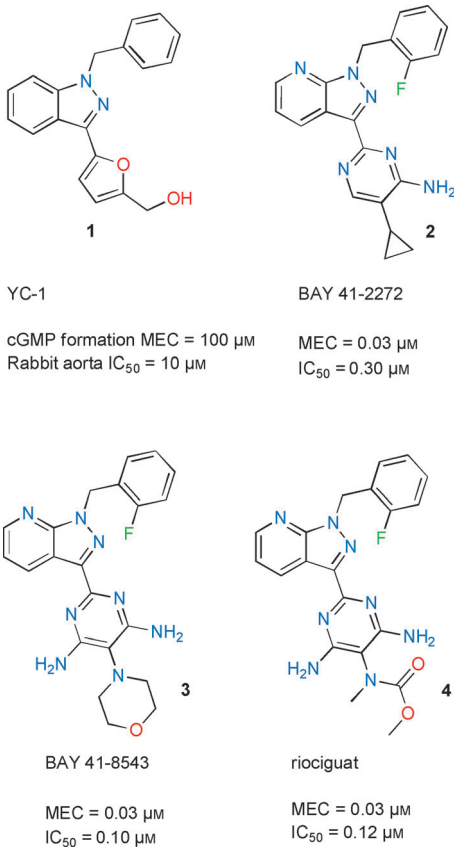
Metabolite identification studies of BAY 41-2272 and BAY 41-8543 revealed an oxidative metabolism at the cyclopropyl and morpholino substituent, respectively.[64] It could be further demonstrated that, in contrast to compounds with small, lipophilic substituents at the pyrimidine C5 position (BAY 41-2272), derivatives with larger, more polar 5-substituents displayed no relevant CYP inhibition (BAY 41-8543). Continuous efforts to introduce other polar, potentially more stable substituents at the pyrimidine C5-position culminated in the identification of the *N*,*O*-dimethylcarbamate **4** (Scheme 1), BAY 63-2521 (International Nonproprietary Name (INN): riociguat).[60]

Riociguat showed no relevant CYP interaction and a superior pharmacokinetic profile, including good oral bioavailability across different species.

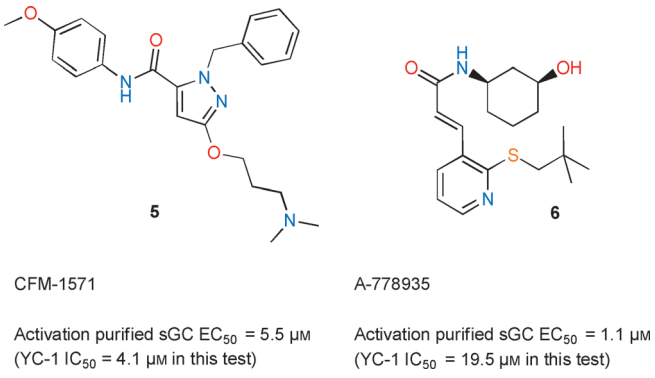
In vitro, riociguat stimulated purified recombinant sGC up to 73-fold, from 0.1 to 100 μM, and showed the typical profile of sGC stimulators: strong synergistic enzyme activation when combined with NO-releasing agents and crucial dependency on the presence of the reduced prosthetic heme moiety.[65]

In conscious, spontaneously hypertensive rats, oral administration of riociguat resulted in a long-lasting and dose-dependent blood pressure decrease. Importantly, and in contrast to nitrates, the effect is preserved over several weeks or when the rats are rendered nitrate-tolerant. In particular, riociguat was investigated in different animal models of pulmonary hypertension (PH), including mice subjected to chronic hypoxia and rats injected subcutaneously with monocrotaline.[65–67] The pyrrolizidine alkaloid monocrotaline is the main toxic principle of *Crotalaria spectabilis* and is used to induce experimental pulmonary hypertension.[68] In these experimental models, riociguat improved pulmonary hemodynamics and prevented, and even partially reversed, features of adverse structural remodeling such as right ventricular hypertrophy and muscularization of small pulmonary arteries.[66] Based on its combined profile of excellent potency, specificity, efficacy, and safety, riociguat was selected as a drug development candidate for the treatment of different forms of pulmonary hypertension (PH).

PH is a severe, progressive, and life-threatening disorder in which the pressure in the pulmonary arteries is significantly increased owing to vasoconstriction and which can lead to heart failure and death. Patients with PH develop a markedly decreased exercise tolerance and reduced quality of life. The most common symptoms of PH include shortness of breath,



Scheme 1. sGC stimulators YC-1 (**1**), BAY 41-2272 (**2**), BAY 41-8543 (**3**), and riociguat (**4**).



Scheme 2. sGC stimulators CFM-1571 (**5**) and A-778935 (**6**).

fatigue, dizziness, and fainting, all of which are worsened by exertion.[69]

According to the clinical classification of PH (Dana Point), there are five different types of PH based on underlying causes, which are pulmonary arterial hypertension (PAH), pulmonary hypertension owing to left heart disease (e.g., PH-LVD), pulmonary hypertension owing to lung disease and/or hypoxemia (e.g., PH-COPD or PH-ILD), chronic thromboembolic pulmonary hypertension (CTEPH), and pulmonary hypertension with unclear multifactorial mechanisms.[70,71] Currently available pharmacological treatments are only approved to treat one of the five types of PH, namely pulmonary arterial hypertension.[69]

Riociguat is the first sGC stimulator to have made a successful transition from animal experiments to controlled clinical studies in patients. In the recently presented randomized, double-blind, placebo-controlled Phase III trials in patients with the PH subforms pulmonary arterial hypertension (PAH) and chronic thromboembolic PH (CTEPH), riociguat met the primary endpoint in exercise capacity (6-minute-walking-distance, 6MWD).[72–75] Riociguat showed a significant improvement in the 6MWD versus the placebo (+36 m, PAH; +46 m, CTEPH; Figure 3). Additionally, improvements were observed across secondary endpoints, including pulmonary hemodynamics, functional class, and time to clinical worsening. Remarkably, riociguat is the first drug that has consistently demonstrated efficacy in two life-threatening PH indications: CTEPH and PAH.

Besides PAH and CTEPH, riociguat also showed clinical effects in proof-of-concept studies in patients with PH secondary to left heart failure, interstitial lung disease, and chronic obstructive pulmonary disease.[67,73] These promising results suggest that sGC stimulators may constitute a valuable new therapy approach for the different types of PH.

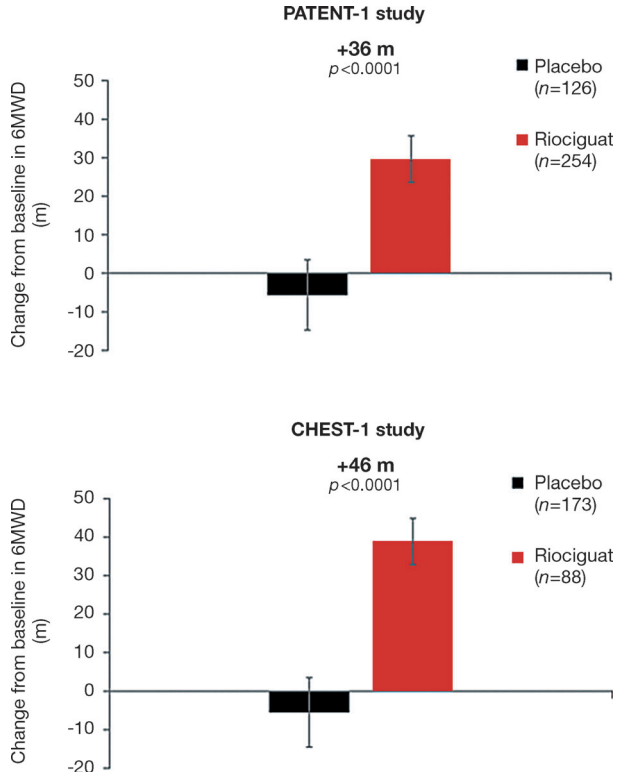
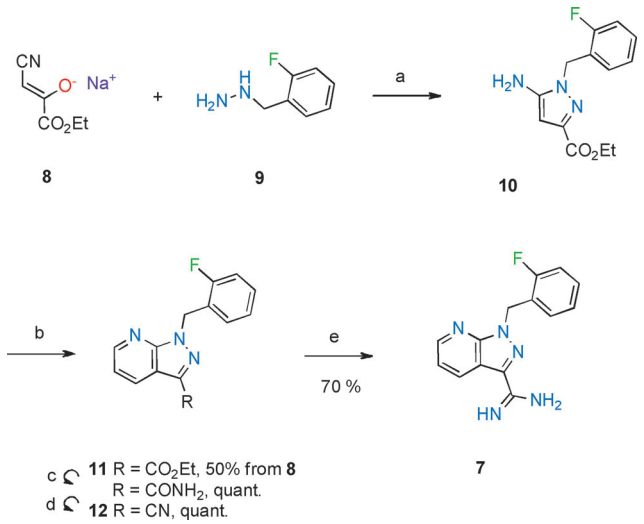


Figure 3. Mean change in the 6-minute-walking-distance (6MWD); primary endpoint at week 12 ($p < 0.0001$) in PATENT-1 (PAH) and at 16 weeks ($p < 0.0001$) in CHEST-1 (CTEPH). Bars represent group means and standard errors.[73]

2.4. Synthesis of sGC Stimulators

The amidine intermediate **7** (Scheme 3) served as a common precursor for the synthesis of BAY 41-2272, BAY 41-8543, riociguat, and many other analogues. For the syn-



Scheme 3. Synthesis of intermediate **7**. Reagents and conditions: a) TFA, dioxane, reflux; b) 3-(dimethylamino)acrolein, TFA, reflux; c) NH_3, MeOH, RT; d) TFAA, Py, RT; e) 1. NaOMe, MeOH, RT; 2. NH_4Cl, AcOH, reflux.

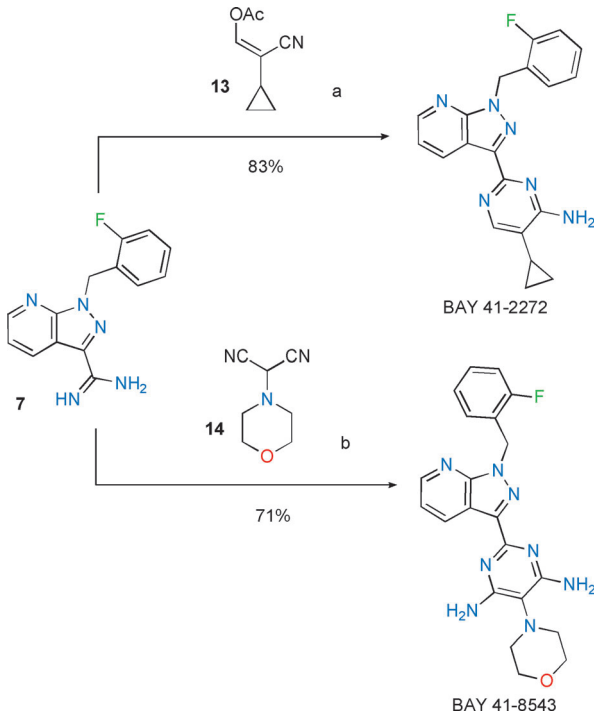
thesis of amidine **7**, the aminopyrazole **10** was generated from ethyl cyanopyruvate (**8**) and 2-fluorobenzylhydrazine (**9**). Subsequent cyclocondensation of the crude product with 3-(dimethylamino)acrolein yielded pyrazolopyridine **11** in 50 % overall yield. The carboxylic ester moiety was subsequently transformed into a nitrile group via classical amide formation and dehydration, to provide **12**. A Pinner reaction of the cyano group of **12** with sodium methanolate and subsequent substitution with ammonia provided amidine **7** in a 35 % total yield over five steps from readily available starting materials.

Monoamino-substituted pyrimidines were prepared by reaction of amidine **7** with differently activated acrylonitrile building blocks, such as enol acetate **13** in the case of BAY 41-2272 (Scheme 4). BAY 41-8543 and other diamino-substituted pyrimidines were obtained via condensation of amidine **7** with substituted malonodinitriles (for example, **14**).

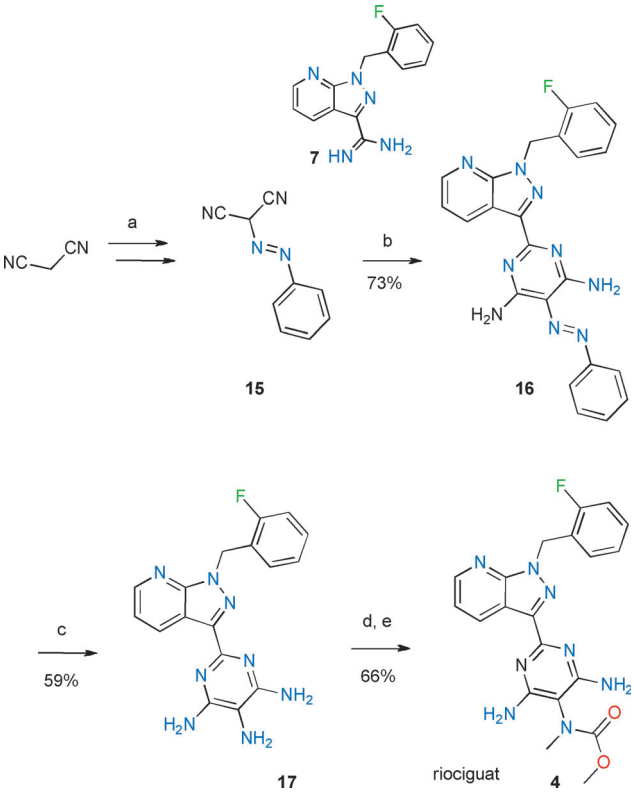
For the preparation of a 4,5,6-triaminopyrimidine precursor of riociguat, phenyldiazo-substituted malonodinitrile **15** (which was first described by Hantzsch in 1905)[76] was condensed with amidine **7** in the presence of sodium methanolate in *N*,*N*-dimethylformamide to provide 5-(phenyldiazo)pyrimidine **16** in 73 % yield (Scheme 5). Subsequent reduction using Raney nickel afforded the 4,5,6-triaminopyrimidine **17**. The 5-amino group in **17** selectively reacted with methyl chloroformate to give the corresponding carbamate in high yield. Further N-methylation using iodomethane (after deprotonation with LiHMDS) provided the *N*-methylcarbamate riociguat (**4**). This sequence turned out to be safe and appropriate for scale-up, and has been used with slight modifications to produce riociguat on a multi-100 kg scale.

2.5. Activities towards Next-Generation sGC Stimulators

Based on an increasing knowledge associated with this mode of action, the promising pharmacological effects of sGC



Scheme 4. Synthesis of BAY 41-2272 and BAY 41-8543. Reagents and conditions: a) toluene, reflux; b) neat, 100 °C.



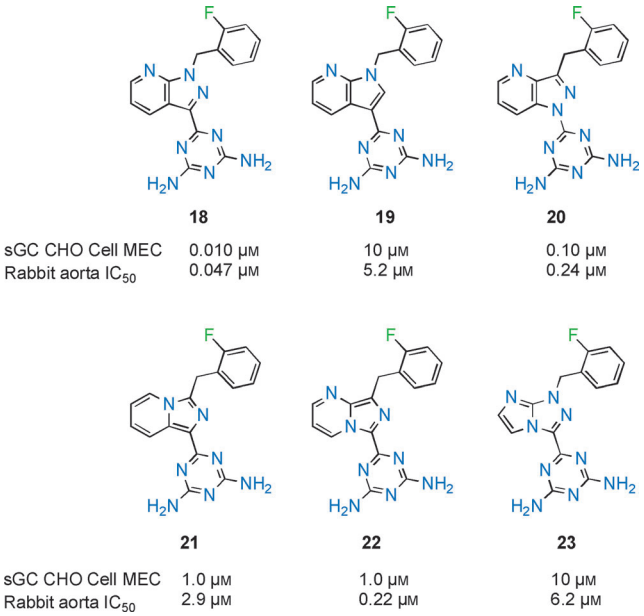
Scheme 5. Synthesis of riociguat (**4**). Reagents and conditions: a) $PhNH_2$, $NaNO_2$, NaOAc, aq. HCl, ethanol, 0 °C; b) NaOMe, DMF, 110 °C, c) H_2, Raney nickel, 65 bar, DMF; d) $ClCO_2Me$, pyridine, 0 °C to RT; e) LiHMDS, THF, 0 °C; then MeI, 0 °C.

stimulators and the clinical success of riociguat, several companies started programs to further explore the structure-activity relationships (SAR) of the pyrimidino-substituted pyrazolopyridines or to identify new lead series of sGC stimulators.

2.5.1. *Modification of the Pyrazolopyridine Moiety*

Optimization of the core framework by bioisosteric replacement is a common approach in medicinal chemistry.[77] For rapid analoging, it is indispensable to identify essential elements of the core motif that must be retained and, ideally, the focus is on simple modifications which can be assessed in short sequences.

The 2,4-diamino-1,3,5-triazine moiety, as in the highly potent compound **18** (Scheme 6), can be accessed in a single synthetic transformation from a cyano or carboxylic ester group and is thus significantly more suited for the purpose of

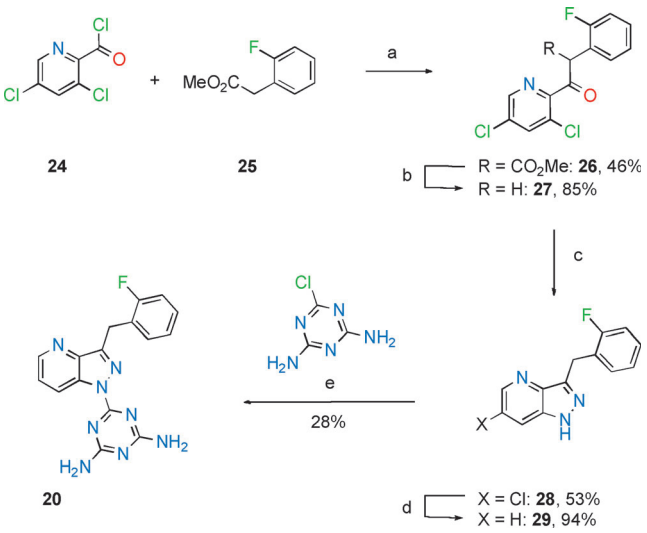


Scheme 6. Modification of the pyrazolopyridine core.

rapid scaffold optimization than the *N*-alkoxycarbonyl-substituted triaminopyrimidine moiety, as in riociguat, which requires seven synthetic steps from ester **11** (Scheme 3, Scheme 5).

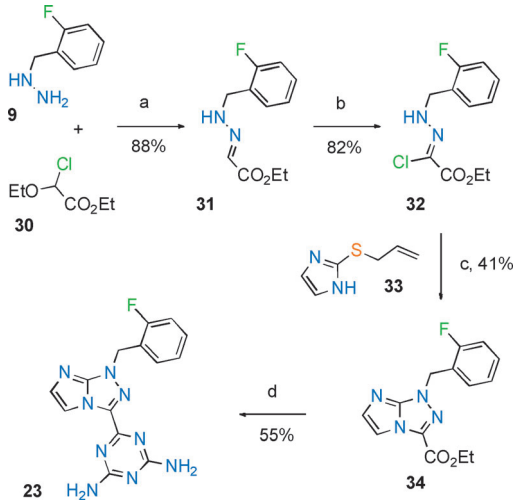
To discriminate essential from nonessential elements of the core fragment, specific elements were consecutively omitted. For example, replacement of a single ring nitrogen, leading to 7-azaindole **19**, resulted in almost complete loss of activity. Flipping the five-membered ring of the pyrazolopyridine core and thus attaching the benzyl substituent to a carbon instead of a nitrogen atom, as in **20**, resulted in 5- to 10-fold less potent sGC stimulation.[78] Further shift of the nitrogen atom into the bridging position, exemplified by **21**, led to a 10-fold loss of potency compared to **20**. Interestingly, the related core fragment of **22** leads to a more potent analogue in the functional assay. Compound **23**, as a representative example of analogues with the 5,5-membered fused heterobicyclic core fragment, is fairly inactive.[79]

The synthesis of 1*H*-pyrazolo[4,3-*b*]pyridine **20** started with the reaction of acid chloride **24** and phenylacetate **25**, giving rise to β-keto ester **26** (Scheme 7). After decarboxylation to **27**, the cyclization with hydrazine in pyridine as solvent in the presence of catalytic amounts of 4-DMAP provided the pyrazolopyridine **28**. The chlorine substituent was removed by hydrogenolysis and the diaminotriazine substituent was attached to the resulting pyrazolopyridine **29** via a Buchwald–Hartwig coupling to furnish **20**.[79]



Scheme 7. Synthesis of **20**. Reagents and conditions: a) LiHMDS, THF, −78 °C; b) NaCl, H_2O, DMSO, MW, 150 °C; c) $N_2H_4{\cdot}H_2O$, Py, DMAP, MW, 160 °C; d) H_2, Et_3N, Pd/C, EtOH; e) $[Pd_2dba_3]$, XPhos, Cs_2CO_3, toluene, 90 °C.

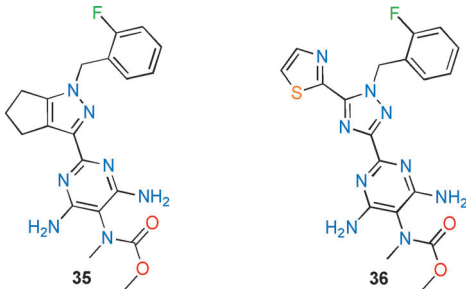
A synthesis to obtain the 5,5-ring-fused derivative **23** (Scheme 8) is based on a 1,3-dipolar cycloaddition strategy via nitrilimines, which are postulated to form from hydrazo-



Scheme 8. Synthesis of **23**. Reagents and conditions: a) aq HCl, dioxane, RT; b) NCS, EtOAc, 60 °C; c) Et_3N, dioxane, reflux; d) biguanide, MeOH, reflux.

noyl halides such as **32** upon treatment with base. The benzylhydrazone **31** of ethyl glyoxylate was prepared via condensation of 2-fluorobenzylhydrazine **9** with ethyl chloro(ethoxy)acetate **30**, and was chlorinated with *N*-chlorosuccinimide to give **32** in high yield. Subsequent reaction with (allylsulfanyl)imidazole **33** furnished bicyclic **34** in 41 % yield, and the final introduction of the triazine head moiety completed the synthesis of **23**.

In 2011, sGC stimulators with saturated ring systems, for example with a 1,4,5,6-tetrahydrocyclopenta[*c*]pyrazole as the central core, were reported by Ironwood Pharmaceuticals (Scheme 9).[80] However, the most active compound **35**, an analogue closely related to riociguat, was reported to be less potent than reference compound BAY 41-2272 in a functional thoracic aortic ring assay.



Scheme 9. sGC stimulators reported by Ironwood Pharmaceuticals.

In a subsequent patent application published in 2012, the same team disclosed further optimized analogues possessing a central 1,2,4-triazole motif with different heteroaromatic moieties as substituents in the 5-position, as exemplified by the thiazole derivative **36** (Scheme 9).[81,82] The most potent derivatives were reported to have an EC_{50} of less than 1 μM in the above-mentioned functional thoracic aortic ring assay.

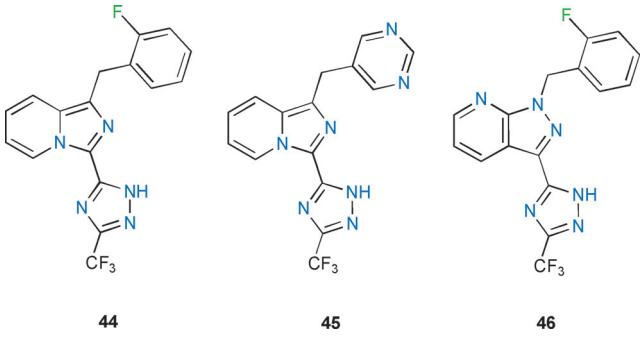
The synthesis of **36** (Scheme 10) started with thiazolecarbohydrazide **37**, which can be condensed with thioamide **38** to form the corresponding 1,2,4-triazole **39**. Subsequent benzylation with **40** yielded an almost 1:1 mixture of regioisomeric triazoles **41** and **42**. After separation, **41** was employed in a reaction sequence that is very similar to the route applied in the synthesis of riociguat (Schemes 3 and 5), finally leading to compound **36**.

So far, no clinical trials of any of these compounds have been reported.

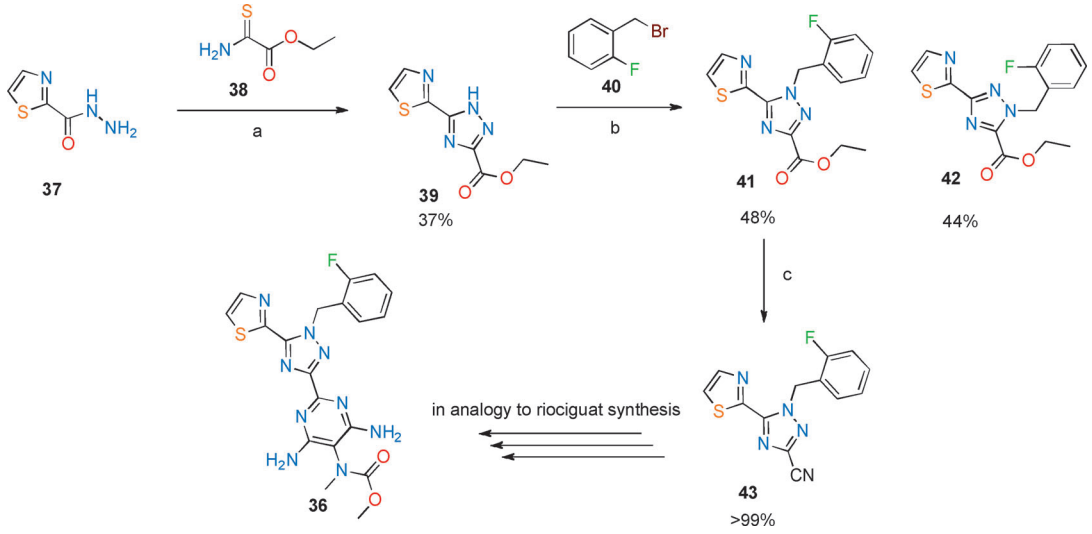
2.5.2. *Replacement of the Aminopyrimidine Group*

Scientists at Pfizer recently described efforts for replacing the aminopyrimidine part of sGC stimulators.[83] Their rational was based on the assumption that introducing an acidic heterocycle as the southern group would improve solubility, while allowing for retained potency and selectivity. Concurrently, they introduced an imidazopyridine instead of a pyrazolopyridine core.

Trifluoromethyl-substituted 1,2,4-triazole **44** (Scheme 11) was found to be about 10-fold less potent than BAY 41-8543 (**3**) in a human sGC cGMP production assay (EC_{10} = 470 nM vs 44 nM for BAY 41-8543) and about 4-fold less potent in a rat aortic ring relaxation assay (40 nM vs 10 nM for BAY 41-8543). A further, improved analogue was identified when the



Scheme 11. 1,2,4-triazoles of Pfizer.

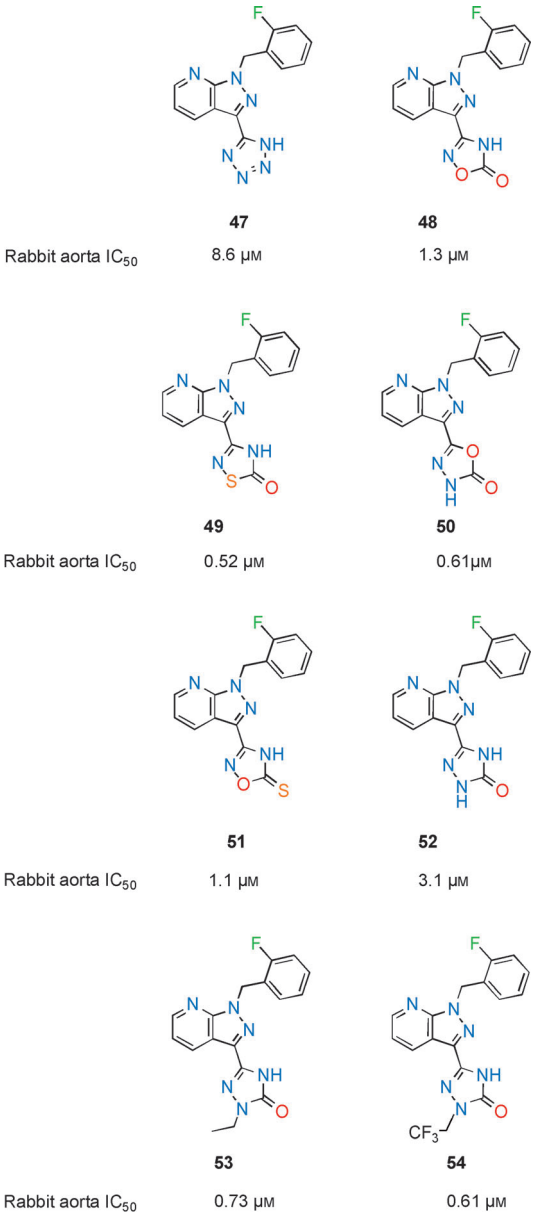


Scheme 10. Formal synthesis of sGC stimulator **36**. Reagents and conditions: a) NH_4Cl, EtOH, 110 °C (sealed tube); b) NaH, DMF, RT; c) NH_3, MeOH, NaCN (cat.), 90 °C (sealed tube).

northern fluorophenyl substituent was replaced by a pyrimidinyl group, leading to triazole **45**, which was slightly less potent in the rat aortic ring assay ($IC_{50} = 60$ nM) but showed improved solubility and stability in human liver microsome incubations.[83]

An analogous triazole **46** with a pyrazolopyridine core was significantly more potent in the cGMP assay ($EC_{50} = 60$ nM) and marginally more potent in the rat aortic ring relaxation assay ($IC_{50} = 33$ nM) than its imidazopyridine counterpart **44**. Triazole **46** was found to be stable in incubations with human liver microsomes; solubility at pH 6.5 was, however, only slightly improved compared to BAY 41-8543 (**3**). No further clinical development of this compound has been reported to date.

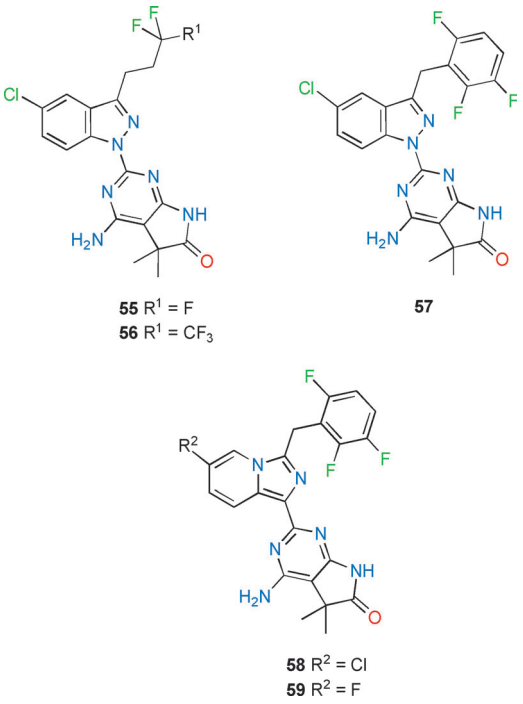
A similar strategy was pursued by Bayer, and the results were recently reported by Griebenow et al.[84] Starting from a tetrazole-containing compound **47** displaying good aqueous solubility albeit low potency,[59] several other five-membered heterocycles were incorporated (Scheme 12). The potency could be increased by one order of magnitude, as shown for thiadiazolone **49**, oxadiazolone **50**, and triazolones **53** and **54**; however, only very few analogues were characterized by significantly improved aqueous solubilities in the range of > 200 mg L^{-1}.



Scheme 12. sGC stimulators reported by Bayer.

2.5.3. *Recent Developments from the Patent Literature*

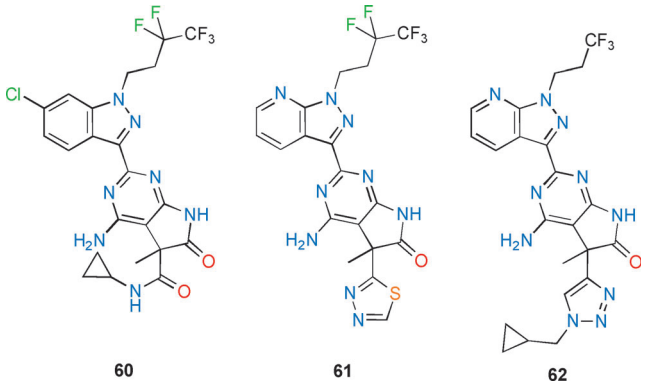
A different approach towards modified sGC stimulators was reported in 2010 by scientists at Merck.[85] They disclosed pyrimidines with a fused dimethylpyrrolidin-2-one ring system, as exemplified by the compounds in Scheme 13. Here, different lipophilic head groups were employed (for example, 2,3,6-trifluorobenzyl and the highly fluorinated 3,3,4,4,4-pentafluorobutyl).



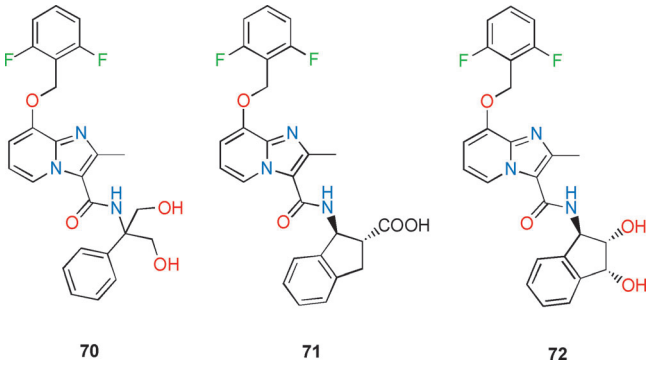
Scheme 13. First sGC stimulators reported by Merck.

In a further development of these compounds, the same team reported derivatives with increased polarity, as exemplified by the sGC stimulators in Scheme 14. Here, one of the methyl groups of the pyrrolidin-2-one system was replaced by an amide or a small heteroaromatic group.[86]

The synthesis of compound **60** started with cyanoindazole **66**, which was built up by standard methodology in five steps, beginning with aniline **63** (Scheme 15). After alkylation of **66** with 1,1,1,2,2-pentafluoro-4-iodobutane, the cyano group of **67** was transformed to an amidine using a trimethylaluminum-mediated amination. Amidine **68** was then condensed with diethyl (dicyanomethyl)(methyl)malonate to form annelated five-membered lactam **69** in one step. Aminolysis of the ester in **69** finally yielded sGC stimulator **60** as a racemic mixture.



Scheme 14. Latest sGC stimulators reported by Merck.



Scheme 16. Imidazo[1,2-*a*]pyridines described by Astellas.

Compound **60** was reported to exhibit maximum systolic blood-pressure lowering effects of minus 77 mmHg when dosed orally to spontaneously hypertensive rats at a dose of 0.3 mg kg^{-1}.[86] So far, a progression of any of these compounds to clinical studies has not been reported.

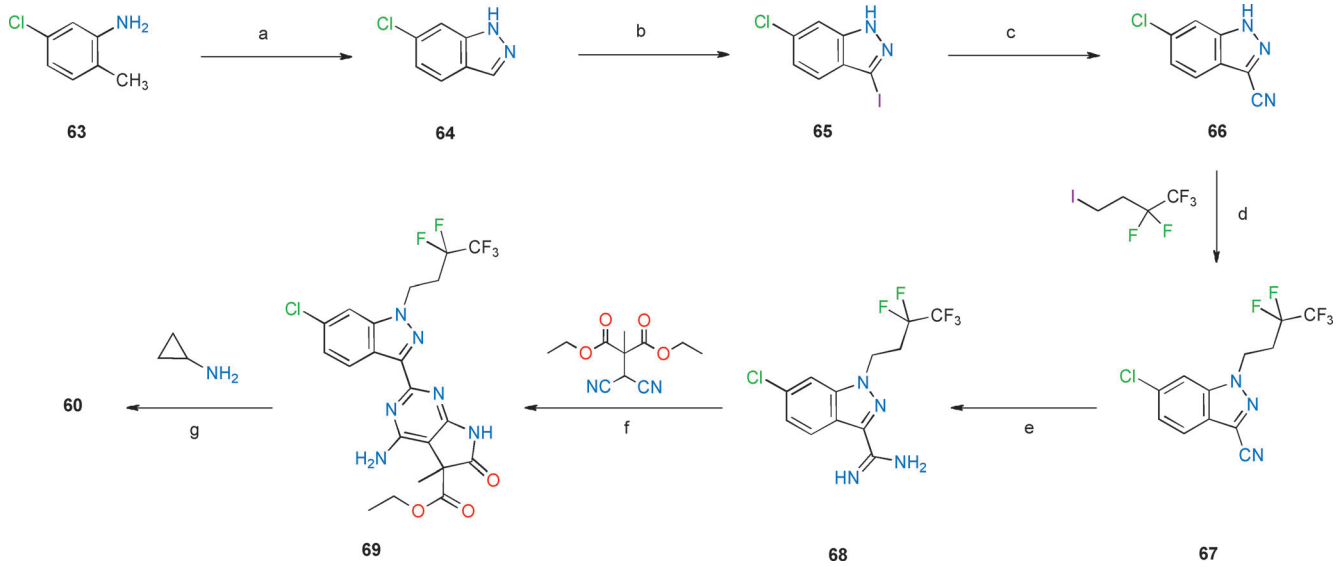
With the exception of the weakly potent cinnamyl amides, so far all classes of sGC stimulators are structurally related to Bayer's first generation of sGC stimulators. However, in 2012, a novel series of sGC stimulators employing an imidazo[1,2-*a*]pyridine scaffold was reported by Astellas (Scheme 16).[87] As a noticeable similarity to the pyrazolopyridine sGC stimulators previously reported, these compounds bear a fluorinated benzylic head group. In this series, a 2,6-difluorobenzyloxy substituent is apparently preferred to gain potency. More than 800 amide derivatives of the imidazo-[1,2-*a*]pyridines have been reported. Future studies have to show how these novel sGC stimulators compare to the pyrazolopyridines such as riociguat.

3. sGC Activators

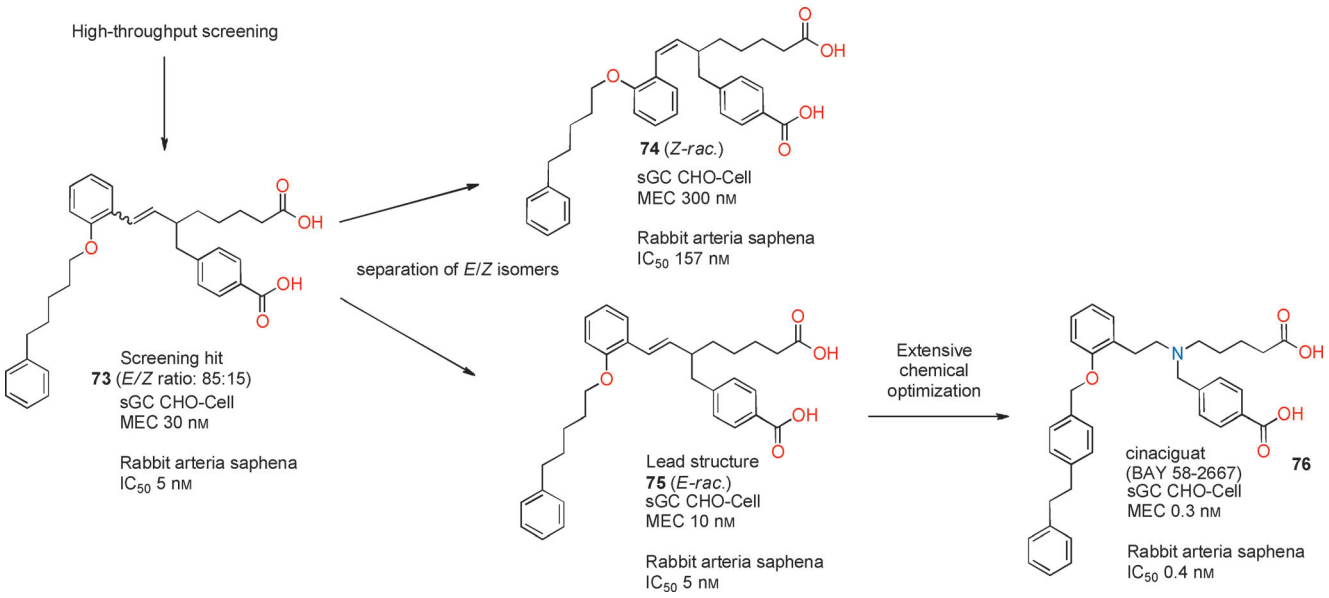
3.1. Discovery of sGC Activators

Following the discovery of the NO-independent, heme-dependent sGC stimulators, scientists at Bayer performed a high-throughput screen (HTS) in 1997 with the goal to identify additional sGC stimulator leads. For this effort, the cGMP formation assay in sGC overexpressing CHO cells was utilized.[58] Surprisingly, an unprecedented and distinct dicarboxylic acid motif **73** was identified, which potently activated sGC (Scheme 17). After further mechanistic in vitro studies, it was established that this compound behaved in a completely different manner to the sGC stimulators, activating sGC in a NO- as well as heme-independent fashion. Thus, the novel compound class was denominated sGC activators.

This serendipitous discovery provided a way to further elaborate on the rationale that the bioactivity of sGC is redox regulated and that this may be key to the pathogenesis of several cardiovascular disorders. More specifically, this offered the prime opportunity to design drugs for selective



Scheme 15. Synthesis of the sGC stimulator **60** of Merck. Reagents and conditions: a) 1. Ac_2O, KOAc, benzene, RT; 2. *t*BuONO, 80 °C; 3. LiOH, MeOH, THF, H_2O, RT; b) NIS, MeCN, 60 °C; c) $Zn(CN)_2$, Zn powder, $[Pd_2dba_3]$/dppf (cat.), DMA, 120 °C; d) K_2CO_3, MeCN, reflux; e) NH_4Cl, $AlMe_3$, toluene, RT to 110 °C; f) $KHCO_3$, *i*PrOH, 80 °C; g) MeOH, 50 °C.



Scheme 17. Discovery of the new class of sGC activators via HTS using sGC overexpressing CHO reporter cells.

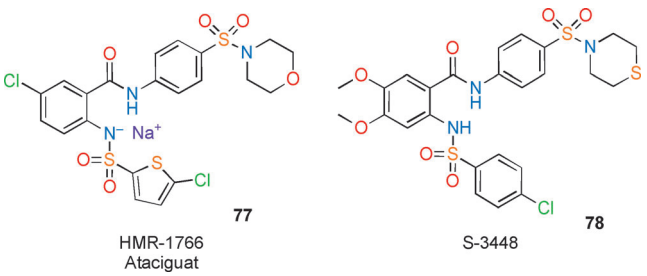
binding to the oxidized, heme-free sGC generated by the influence of oxidative stress causally involved in many cardiovascular diseases.[27]

Screening hit **73** presented as a racemic 85:15 *E*/*Z*-mixture. After separation, the racemic *E*-isomer **75** turned out to be 30-fold more potent than the racemic *Z*-isomer **74**. Subsequent separation of enantiomers revealed that the *R*,*E*-isomer of **75** is 70-fold more potent than the corresponding *S*-enantiomer. Moreover, lead structure **75** also showed promising in vitro potency in isolated recombinant sGC and relaxation of precontracted rabbit arteria saphena rings.[88] Based on these initial results, an extensive lead optimization program was started with the goal to identify a candidate suitable for intravenous dosing. The exchange of the central allylic moiety for an ethylamino linkage and modification of the phenylpentyl side chain resulted in the discovery of clinical candidate BAY 58-2667 (**76**; INN: cinaciguat).[46,89–94]

The pharmacological efficacy profile of cinaciguat was explored in various in vivo models for myocardial infarction, chronic renal failure, arterial and pulmonary hypertension, and chronic heart failure. In a canine model of congestive heart failure (HF), intravenous administration of cinaciguat resulted in dose-dependent reductions in cardiac preload and afterload, and a concomitant increase in cardiac output and renal blood flow without further neurohumoral activation.[95]

Based on these promising results, a nonrandomized, unblinded, multicenter Phase IIa study in patients with acute decompensated heart failure (ADHF) was initiated. Continuous intravenous infusion of cinaciguat was well-tolerated and resulted in an improvement of cardiopulmonary hemodynamics similar to the described canine model of congestive heart failure. The subsequent clinical Phase IIb program studied the effects of cinaciguat in three randomized, double-blind, placebo-controlled studies in ADHF patients;[96] however, the clinical development of cinaciguat had to be stopped owing to difficulties in controlling blood pressure reduction, which might be unfavorable in patients with ADHF.[97–99]

In 2001, researchers from Hoechst Marion Roussel disclosed anthranilic acid derivatives, which represented a novel structural class of compounds also reported to activate the oxidized and/or heme-free form of sGC.[100] The best-described examples are HMR 1766 (**77**; INN: ataciguat) and S-3448 (**78**; Scheme 18).



Scheme 18. sGC activators reported by Hoechst Marion Roussel: ataciguat (**77**) and S-3448 (**78**).

The inhibition of phenylephrine-induced contraction of rat aortic rings by both compounds is only moderate to weak ($IC_{50} = 0.46$ μM and 0.52 μM, respectively);[101] however, the pharmacological efficacy of ataciguat and S-3448 was demonstrated in various in vivo models of atherosclerosis and peripheral arterial occlusive disease. Chronic treatment of streptozotocine diabetic rats with ataciguat improved endothelial function and normalized platelet activation.[102] Additionally, reduced atherosclerosis and improved endothelium-dependent vasorelaxation was observed in $ApoE^{-/-}$ mice treated with ataciguat.[103]

Stage II peripheral arterial occlusive disease is mainly characterized by exercise-induced muscle fatigue. Ataciguat improved ischemia-induced muscle fatigue in zucker diabetic

fatty (ZDF) rats with unilateral hind-limb ischemia as an experimental model of peripheral arterial occlusive disease.[104]

Based on these data, ataciguat was studied in a Phase II clinical trial for patients with peripheral arterial occlusive disease. So far, the results have not been published, but in February 2009 the study was discontinued without further explanation.

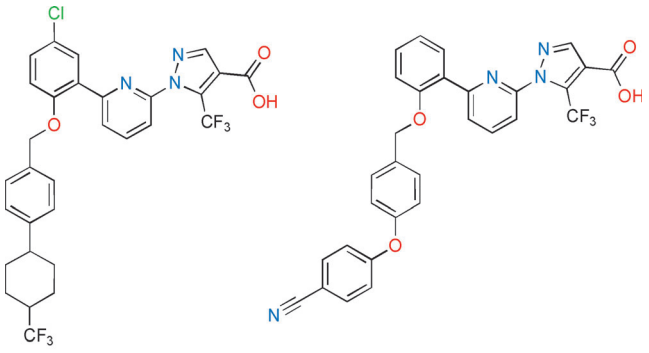
Additionally, ataciguat was investigated in a Phase II study in neuropathic pain patients; however, its clinical development has seemingly been stopped.

3.2. Activities towards Next-Generation sGC Activators

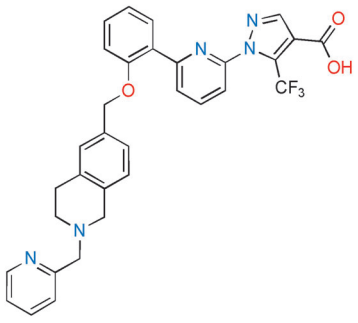
In the last few years, the search for novel sGC activators has become an increasingly competitive field. Several approaches have been reported in the recent patent literature. Interestingly, all second-generation sGC activators contain a monocarboxylic acid moiety.

In 2009, Merck[105] (compound **79**) and GlaxoSmithKline (GSK)[106] (compound **80**) disclosed very similar sGC activators incorporating an identical 5-(trifluoromethyl)pyrazole-4-carboxylic acid moiety attached to a pyridine scaffold (Scheme 19). Even more recently, Boehringer Ingelheim disclosed similar 5-(trifluoromethyl)pyrazole-4-carboxylic acids (compound **81**) displaying high in vitro potency.[107]

Two further series of sGC activators were described in 2010 by GSK, consisting of thiazolylpiperidinecarboxylic



79 80



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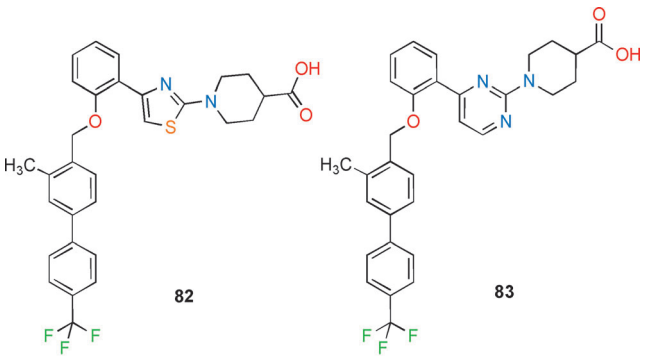
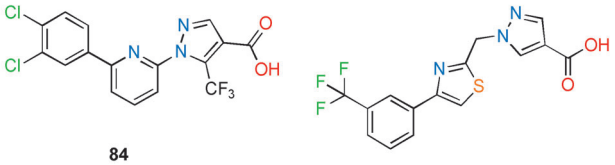
Scheme 19. sGC activators described by Merck, GSK, and Boehringer Ingelheim.



82 83



84 85

Scheme 20. Further sGC activators from the patent literature, reported by GSK and Takeda.

acids[108] (such as **82**) and pyrimidinylpiperidinecarboxylic acids[109] (such as **83**), respectively (Scheme 20). The in vitro potency is reported to be in the range of the above-noted compounds.

Furthermore, compounds with a shorter, less lipophilic tail group were reported in patent applications by GSK[110] (such as **84**) and Takeda (such as **85**).[111] Overall, these compounds have a weaker in vitro potency; however, they might have improved metabolic and pharmacokinetic (DMPK) properties owing to reduced molecular weight.

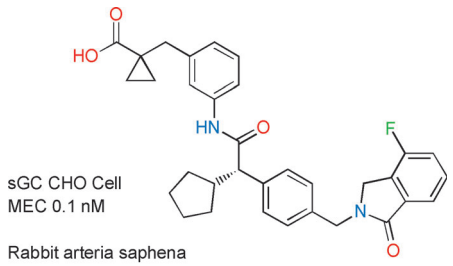
Bayer has also disclosed monocarboxylic acids with novel structural features, highlighting branched 3-phenylpropionic acid derivatives, as exemplified by compound **86** (Scheme 21).[112,113] With the aim of improving the DMPK profile of these compounds, lower molecular weight 3-phenylpropionic acid congeners have been prepared, as exemplified by compound **87**.[114–116]

So far, progression of any of these new sGC activators into clinical trials has not been reported.

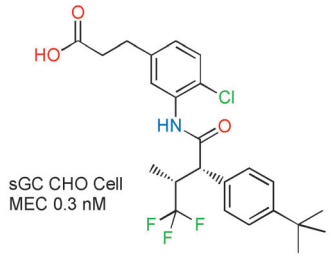
4. Structural Biology

4.1. Structural Biology of sGC and Current Understanding of its Activation Mechanism

Owing to a lack of target X-ray structures, optimization of sGC stimulators and activators solely relied on iterative cycles of synthetic medicinal chemistry and pharmacological and pharmacokinetic testing, without guiding input from computer-aided ligand design. Over the last decade, efforts from various research groups have shed some light on the structural biology of sGC and its activation. The recent elucidation of the binding mode of the sGC activator cinaciguat to its target



86



87

Scheme 21. 3-Phenylpropionic acid sGC activators disclosed by Bayer.

(see Section 4.3) finally opened the door for the study of the sGC activation on a molecular level.[117]

sGC is a heterodimeric enzyme comprised of an α- and a β-subunit (Figure 4).[118] The most abundant subunit isoforms are α1 (encoded by the human *GUCY1A3* gene) and β1

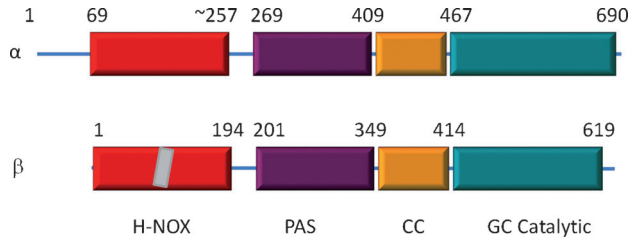


Figure 4. Domain architecture of heterodimeric sGC; the α-subunit is comprised of 690 amino acids and the β-subunit is comprised of 619 amino acids harboring the heme in the H-NOX domain (gray box).

(encoded by the human *GUCY1B3* gene), but other isoforms exist in various tissues. Knockout studies imply that the α1β1-heterodimer is mainly responsible for the vasodilatory effect of sGC upon stimulation by NO.[119] The α2β1-heterodimer can form additional interactions with protein PSD-95[120] and has a more specific tissue distribution.[121] It was shown that BAY 41-2272 (**2**) is also able to activate this isoform.[122] For cinaciguat (**76**), an isoform-specific activity was measured in vitro: The activating effect on the α1β1-heterodimer was stronger than the effect on α2β1.[121]

Furthermore, a physiological role of the homodimers is emerging. Although they are catalytically inactive, the equilibrium between homo- and heterodimers was postulated to regulate sGC activity.[44] Recent results point to separate effects of the single subunits related to androgen regulation and cell cycle progression.[123,124]

Both subunits are organized in four domains (Figure 4). The β1-subunit consists of a N-terminal heme-binding domain which is closely related to a family of *h*eme-containing and *NO*- and o*x*ygen-binding enzymes and therefore is referred to as the H-NOX domain. The β1-H-NOX domain is the diatomic gas receptor of sGC; apart from NO, also CO but not O_2 bind to the ferrous heme. His105 functions as the fifth ligand for the heme iron and plays a key role in the sGC activation process. Little is known about the N-terminal domain of α1-subunit, but heme binding to this domain is unlikely since almost all heme-anchoring amino acids of the β1-subunit are not present in the α1-subunit.

The H-NOX domain is followed by two domains that according to the current knowledge are important for the formation of a physiologically functional and stable dimer: a domain with a PAS-like fold (named after the three proteins Per/Arnt/Sim), followed by a coiled-coil domain. The C-termini of the α- and β-subunits are homologous to the catalytic domains of adenylate cyclases and are responsible for cyclizing GTP to cGMP. This domain is only catalytically active in a heterodimeric complex as the active site is located at the interface between the two homologous subunits and comprises residues from both the alpha and beta chains.[125–128]

Although a crystal structure for the full-length α- or β-subunit of sGC is not available, most domains of related proteins or of sGC itself have been crystallized and provide valuable insights into the organization and activation process of sGC (see Figure 5 for a depiction of the available structural information as well as a possible subunit arrangement).[125,127–131] Crystal structures of the PAS domain of STHK protein[125] and of the coiled-coiled domain of rat sGC[129] showed that both domains seem to be involved in heterodimerization of sGC. Further studies indicate direct interactions of the catalytic domain with the H-NOX domain.[132,133] These findings were recently supported by 3D modeling studies based on small angle X-ray scattering and chemical cross-linking experiments.[134]

Only days before the completion of this Review, the first crystal structure of a mutated heterodimeric catalytic domain of human sGC was published by—*nomen est omen*—the Structural Genomics Consortium (SGC) in Oxford/UK.[128]

The solved X-ray structure at 2.08 Å resolution of the α1/β1 heterodimeric catalytic domain comprises a dimer reminiscent of previously solved mammalian adenylate cyclases and guanylate cyclases from bacteria and algae.[127,131] Overall, the α1-β1-heterodimer is likely in an inactive conformation. Alignment with an adenylate cyclase active conformation shows the need for significant rearrangements of the sGC heterodimer upon activation, with movements of up to 10 Å for individual residues.

For the H-NOX domain, the X-ray structure of the human isoform is not available; homologues from different bacteria strains have however been evaluated structurally.[40,41,130] The most similar H-NOX domain to human sGC is that of *Nostoc sp.* (*Ns* H-NOX), which shares a 35% sequence identity. In the NO-free (inactivated) structure, the heme moiety is sandwiched between a small α-helical and a larger mixed-α/β-subdomain. A conserved histidine (His[105]) functions as the fifth ligand for the five-coordinate iron.

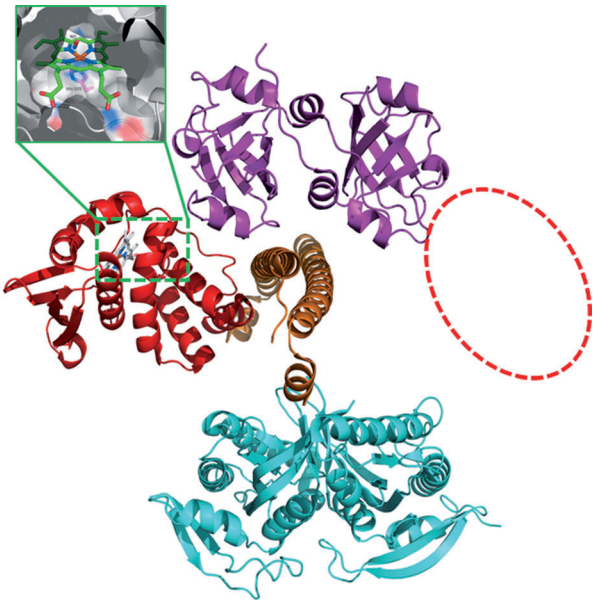


Figure 5. A possible 3D arrangement of all structurally known domains derived from X-ray structures of sGC or homologues enzymes is shown: β-H-NOX domain in red with bound heme (PDB code 2O0C),[130] PAS domain in purple (2P04),[125] CC domain in orange (3HLS)[129] and catalytic domain in cyan (3UVJ).[128] The α-H-NOX domain is shown as a blank red ellipsoid, as no sufficiently homologous structure is available in the protein data bank.[48] A zoom of the binding site of the heme in the β-H-NOX domain is highlighted in the green box.

Prerequisite for full sGC activation is the binding of NO to the ferrous (Fe^{2+} oxidation state) heme to form an intermediate six-coordinate complex and subsequent dissociation of His105 to a five-coordinate state. However, more recent studies argue that a formal dissociation may not be required to trigger signal transduction.[135] The conformational change in the H-NOX domain is propagated to the heterodimeric catalytic domain where cGMP production is subsequently enhanced. Strain relaxation of the heme cofactor upon His105 dissociation (or lengthening of the Fe–His bond) being coupled to N-terminal movements[136] has been proposed as link between NO binding and sGC activation ("loaded spring" hypothesis of sGC activation).[137]

The dissociation of His105 upon NO binding is an example for the thermodynamic *trans* influence, which describes the tendency of a ligand to weaken the bond *trans* to itself.[138] This effect reflects competition of σ-donor orbitals of NO and His105 for the d_{z^2} iron orbital with NO as the stronger donor weakening in turn the binding strength of the weaker donor His^{105}.[139] Of note, CO also binds to ferrous heme but as it is a significantly weaker σ-donor than NO is it not able to execute a sufficiently strong *trans* influence to induce dissociation of His^{105}.[140,141] Therefore, CO causes only low levels of sGC activation.

Along with the *trans* influence of NO (by some authors also referred to as "negative *trans* effect"), other structural features around the heme binding site are likely causative for the unusual ability of sGC (compared to other heme containing enzymes) to break the heme–His bond upon NO binding. Release of tension of neighboring side chains on His105 may be one of these additional influencing factors.[142] A more detailed discussion of these factors and studies on related heme-containing enzymes is beyond the scope of this Review.

An additional level of complexity arises from evidence towards the existence of a second lower-affinity binding site for NO. Some research groups have demonstrated that the conversion of the hexacoordinated intermediate state into the five-coordinate active nitrosyl–heme complex depends on the concentration of free NO, suggesting that there is a second binding site for NO.[118,143–145]

Stoichometric binding of NO to the heme cofactor leads to low-level enzyme activation. A much higher catalytic activity is achieved when either additional NO or sGC stimulators, such as YC-1 (**1**) or BAY 41-2272 (**2**), are available.[143,146] Furthermore, an allosteric modulation by nucleotides (for example, GTP, ATP) of the duration and intensity of enzyme activation was observed.[147] Amongst the many spectroscopic studies that discuss details of the NO heme interaction,[148,149] some have studied the structural changes imposed on the enzyme by the binding of sGC stimulators. Using resonance Raman spectroscopy, no spectral difference between the binding of the first NO to heme and the presence of excess NO could be observed. In contrast, significant changes appeared when sGC stimulators were present, presumably because of a change in heme geometry and a small modification of the Fe-N-O angle.[146]

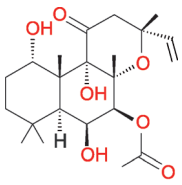
Studies that analyze the interaction between carbon monoxide (CO), YC-1 (**1**), and sGC further support the indirect effect of sGC stimulators. Although CO binding stimulates sGC only weakly, it could be shown with sGC of different species that CO binding enables enzyme activation by YC-1 (**1**) to an extent comparable to that of NO binding.[27,55,150] A proposed mechanism is the reduction of NO and CO off-rates by YC-1 (**1**).[150] The effect was not only observed in vitro but also in various tissues, such as pig urethra, rat aortic strips, and human platelets.[151–153]

4.2. Studies to Elucidate the Structural Basis for sGC Stimulation

To date, no X-ray data elucidating the binding site and mode of action of sGC stimulators are available. Alternative experimental approaches attempting to locate the binding site of sGC stimulators (for example, binding to sGC mutants, Raman spectroscopy, or photoaffinity labeling studies with designed analogues) have provided seemingly conflicting data.

Based on UV/Vis spectroscopic studies, a direct interaction of sGC stimulators, such as BAY 41-2272 (**2**), with the heme prosthetic group was excluded.[154] More recently, resonance Raman spectroscopic investigations have suggested that addition of YC-1 or BAY 41-2272 (**2**) to NO-bound sGC induces a change in the heme geometry.[146] This change may reflect the stimulator-induced shift to a more active sGC conformation and does not necessarily reflect a direct interaction of the ligand with the heme group.

The dimeric nature of ACs and sGCs results in formation of a pseudosymmetric second cavity in their catalytic domains. This cavity is catalytically inactive as it lacks key residues which are believed to be relevant for cGMP or cAMP formation. In case of ACs, the pseudosymmetric site is the binding site of the known allosteric AC stimulator forskolin (see Figure 6 for its chemical structure), a diterpene produced by the indian coleus plant *coleus forskohlii*.[155]



Forskolin

Figure 6. Chemical structure of the known adenylate cyclase (AC) stimulator forskolin.

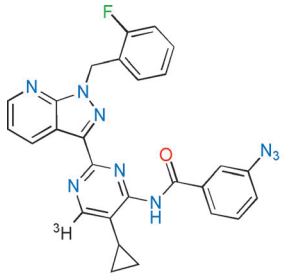
The forskolin binding site on ACs has stimulated the hypothesis of the presence of a similar binding site in the sGC catalytic domain to which allosteric sGC stimulators may bind, despite the absence of any structural similarities between forskolin and the known sGC stimulator classes. Mutational and docking studies with YC-1 were carried out to investigate this hypothesis.[156] Binding studies with nucleotide analogues indeed showed the presence of two nucleotide binding sites on sGC. Furthermore, for YC-1 (**1**) and sGC stimulator BAY 41-2272 (**2**), competition with nucleotide analogues for one of these binding sites was experimentally verified.[157]

In the recently solved first crystal structure of a heterodimeric α1β1-catalytic domain,[128] which is believed to reflect an inactive conformation, the "forskolin-like" binding site is collapsed. However, in the modeled active conformation an additional binding site (smaller than in AC) opens up in which sGC effector molecules may bind.

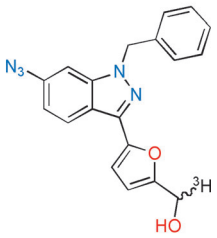
Despite the existence of the two sites in the catalytic domain of sGC, previous studies showed that the activity of the isolated catalytic sGC domains is not influenced by YC-1 (**1**) or BAY 41-2272 (**2**), indicating that the catalytic site is not binding sGC stimulators.[158] This is in line with data showing that a truncated *Manduca sexta* (tobacco hornworm) sGC construct, lacking the catalytic domain, still reacts to sGC stimulators like YC-1 (**1**).

To provide additional data on the mode of action of the sGC stimulators, in situ generation of a highly reactive ligand radical or nitrene in the presence of the protein of interest is a commonly applied approach to map binding sites. A tritiated analogue **88** of BAY 41-2272 (**2**) with a photoaffinity tag attached to the primary amino group was synthesized for this purpose (Scheme 22).[159] Introduction of the tritium label was achieved by synthesizing first a brominated precursor, which was then employed in a palladium-catalyzed debromination reaction under an atmosphere of tritium, followed by acylation with 3-azidobenzoyl chloride, which gave compound **88** with a specific activity of 7.9 Ci mmol^{-1} (292 GBq mmol^{-1}).

Compound **88** showed a sGC stimulatory profile closely resembling BAY 41-2272 (**2**).[159] This result is in line with the general SAR trend that attachment of sterically demanding groups to the pyrimidine amino group is well-accepted. By selecting this site for the attachment of the photoaffinity label and proving that the pharmacological profile compared to BAY 41-2272 (**2**) is unaffected, it is highly likely that



88



89

Scheme 22. Tritiated sGC stimulator **88** and tritiated YC-1 analogon **89** for photoaffinity labeling studies of sGC.

compound **88** binds sGC in a typical stimulator fashion. However, by design, the photoactive azido group is rather distant from the northern and core part of the molecule, for which steep SAR data implicate an especially tight interaction with the sGC enzyme.

Irradiation of compound **88** in the presence of sGC led to an almost exclusive labeling of the α-subunit of sGC, which was significantly diminished in the presence of BAY 41-2272 (**2**), underscoring competition for a common binding site. Fragmentation of the labeled rat protein followed by separation and sequencing showed that compound **88** was bound to Cys238 and Cys243 at the α-subunit. Both amino acids are located either at the C-terminus of the α-H-NOX domain or in the linker region between the α-H-NOX and α-PAS domains.

In a related study, the tritiated photoaffinity analogue **89** of YC-1 (Scheme 22) was irradiated together with sGC.[160] Synthesis of **89** was achieved from azido-YC-1 by oxidation (MnO_2) to the corresponding furancarbaldehyde, followed by reduction with tritiated sodium borohydride. Again, preferential labeling of the α-subunit was observed. However, competition with YC-1 (**1**) was low, thereby pointing to a different binding mode for **89** compared to YC-1 (**1**).[160]

As no crystal structure of full-length sGC is available, it is still possible that both compounds used in this study were actually bound at the interphase between the sGC subunits, thereby selectively labeling the α-subunit despite being bound to the β-subunit. Subsequent mutational studies of the α-subunit showed no effect on the BAY 41-2272-induced activation of sGC, which calls a binding site on the α-subunit into question.[27]

An autoinhibitory interaction between the C-terminal catalytic domain and the N-terminal H-NOX domain has been proposed that may be relieved by excess NO and sGC stimulators binding to or close to the domain interphase.[133]

In conclusion, sGC stimulators exert their effect through an allosteric interaction, resulting in a conformational change that transfers sGC from a low- to a high-level activation state. Whether sGC stimulators indeed bind in a forskolin-reminiscent manner to the catalytic domain of sGC or rather at the interphase between the catalytic domain and the H-NOX domain is still open for debate. Further studies are required for elucidation of the exact binding site.

4.3. Structural Understanding of sGC Activation

Contrary to the situation for the stimulators, where the mode of action is still unclear, a reliable model of how activators act on sGC exists. It was postulated early on that cinaciguat binds to the H-NOX domain of sGC and that cinaciguat and heme compete for this binding site.[41] The heme group of sGC is bound to the β-subunit via its axial ligand His105 and the unique sGC heme-binding motif Tyr135-Ser137-Arg139 (Y-S-R).[41–43,126,161,162] This "competition" hypothesis, based on activity and binding assays, mutational studies, and also spectroscopic studies, was further strengthened by structural alignments showing that cinaciguat is able to mimic the spatial structure of the sGC porphyrin ligand.[42] Valuable insight came from crystal structures of the H-NOX domain of *Nostoc sp.* in complex with cinaciguat[117] and a very close analogue.[163] As predicted, cinaciguat displaces heme from its binding site (Figure 7); upon binding,

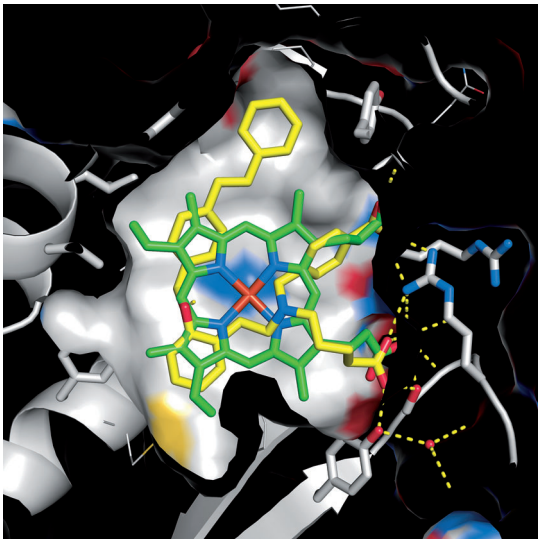


Figure 7. X-ray structure of the β-H-NOX domain *Nostoc sp* with cinaciguat (yellow) superimposed with heme (green). The X-ray structures clearly proved the competitive binding of cinaciguat to heme, as well as the mimicry of the carboxylic acids of cinaciguat to those of the heme.

His105 is displaced by about 0.7 Å from its "inactive" heme-bound conformation. As a consequence, the helix harboring His105 is rotated by about 3°, culminating in surface distortion of a surface region in H-NOX, which is most likely used to transmit the activation signal to the rest of sGC as previously postulated.[133] The structural data were furthermore supported by mutagenesis studies.[117] Interestingly, the close analogue of cinaciguat is more ordered in the crystal structure and therefore has a reduced effect on the overall distortion of the interacting surface region in H-NOX.[163]

In recent years, a deeper insight into the structure of sGC and its activation has been achieved. In particular, the binding and mechanism of action of sGC activators such as cinaciguat is now much better understood. However, many questions remain unanswered, and further studies on the activation and catalytic activity of sGC are of ongoing high relevance for the understanding and subsequent modulation of this key enzyme.

5. Summary and Outlook

Heme-dependent sGC stimulators and heme-independent sGC activators are novel therapeutic options for cardiopulmonary diseases associated with endothelial dysfunction. Riociguat as the most advanced sGC stimulator has been investigated in recent Phase III clinical trials for pulmonary arterial hypertension (PAH) and chronic thromboembolic pulmonary hypertension (CTEPH). Further preclinical and clinical research is ongoing and the future will reveal the full potential of this novel mode of action.

After the initial failures of cinaciguat and ataciguat, sGC activators have recently gained attention, and many new players have entered this field of research. The perspective to specifically activate heme-free sGC in diseases associated with oxidative stress seems very attractive and holds the promise of offering novel therapies for various disorders.

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