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RESEARCH PAPER

Distinct molecular requirements for activation or stabilization of soluble guanylyl cyclase upon haem oxidation-induced degradation

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Background and purpose: In endothelial dysfunction, signalling by nitric oxide (NO) is impaired because of the oxidation and subsequent loss of the soluble guanylyl cyclase (sGC) haem. The sGC activator 4-[((4-carboxybutyl){2-[(4-phenethylbenzyl) oxy]phenethyl}amino)methyl[benzoic]acid (BAY 58-2667) is a haem-mimetic able to bind with high affinity to sGC when the native haem (the NO binding site) is removed and it also protects sGC from ubiquitin-triggered degradation. Here we investigate whether this protection is a unique feature of BAY 58-2667 or a general characteristic of haem-site ligands such as the haem-independent sGC activator 5-chloro-2-(5-chloro-thiophene-2-sulphonylamino-*N*-(4-(morpholine-4-sulphonyl)-phenyl)-benzamide sodium salt (HMR 1766), the haem-mimetic Zn-protoporphyrin IX (Zn-PPIX) or the haem-dependent sGC stimulator 5-cyclopropyl-2-[1-(2-fluoro-benzyl)-1*H*-pyrazolo[3,4-*b*]pyridin-3-yl]-pyrimidin-4-ylamine (BAY 41-2272).
Experimental approach: The sGC inhibitor 1H-(1,2,4)-oxadiazolo[4,3-*a*]quinoxalin-1-one (ODQ) was used to induce oxidation-induced degradation of sGC. Activity and protein levels of sGC were measured in a Chinese hamster ovary cell line as well as in primary porcine endothelial cells. Cells expressing mutant sGC were used to elucidate the molecular mechanism underlying the effects observed.
Key results: Oxidation-induced sGC degradation was prevented by BAY 58-2667 and Zn-PPIX in both cell types. In contrast, the structurally unrelated sGC activator, HMR 1766, and the sGC stimulator, BAY 41-2272, did not protect. Similarly, the constitutively haem-free sGC mutant β_1H105F was stabilized by BAY 58-2667 and Zn-PPIX.
Conclusions: The ability of BAY 58-2667 not only to activate but also to stabilize oxidized/haem-free sGC represents a unique example of bimodal target interaction and distinguishes this structural class from non-stabilizing sGC activators and sGC stimulators such as HMR 1766 and BAY 41-2272, respectively.
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Keywords: soluble guanylyl cyclase; BAY 58-2667; HMR 1766; BAY 41-2272; oxidative stress; nitric oxide; cGMP; haem

Abbreviations: BAY 41-2272, 5-cyclopropyl-2-[1-(2-fluoro-benzyl)-1*H*-pyrazolo[3,4-*b*]pyridin-3-yl]-pyrimidin-4-ylamine; BAY 58-2667, 4-[((4-carboxybutyl){2-[(4-phenethylbenzyl)oxy]phenethyl}amino)methyl[benzoic]acid; CHO, Chinese hamster ovary; EC, endothelial cell; HMR 1766, 5-chloro-2-(5-chloro-thiophene-2-sulphonylamino-*N*-(4-(morpholine-4-sulphonyl)-phenyl)-benzamide sodium salt; HSP90, heat shock protein 90; ODQ, 1H-(1,2,4)-oxadiazolo[4,3-*a*]quinoxalin-1-one; $ONOO^-$, peroxynitrite; PPIX, protoporphyrin IX; RLU, relative light unit; ROS, reactive oxygen species; sGC, soluble guanylyl cyclase; WT, wildtype; Zn-PPIX, zinc-protoporphyrin IX

Introduction

The second messenger cyclic guanosine monophosphate (cGMP), synthesized from guanosine triphosphate (GTP) by soluble guanylyl cyclase (sGC), is a key regulator of vascular smooth-muscle cell relaxation and inhibition of platelet aggregation (Lucas *et al.*, 2000). sGC is a heterodimeric enzyme consisting of an α and a haem-containing β subunit, which represents the intracellular receptor for the gaseous messenger, nitric oxide (NO). However, other mechanisms of sGC regulation have been described such as membrane association or binding to the chaperone heat shock protein 90 (HSP90; Zabel *et al.*, 2002; Agulló *et al.*, 2005; Nedvetsky *et al.*, 2008). The prosthetic haem group is non-covalently bound to

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the β_1 subunit via the proximal haem ligand H105 (Wedel *et al*., 1994; Zhao *et al*., 1998) and the haem-binding motif Y-x-S-x-R (Pellicena *et al*., 2004; Schmidt *et al*., 2004; 2005; Ma *et al*., 2007). Binding of NO to the haem group activates sGC and results in a considerable increase (up to 200-fold) in the formation of cGMP (Ignarro *et al*., 1982). In turn, cGMP affects various downstream targets such as protein kinases, cyclic nucleotide-gated channels or phosphodiesterases (Lucas *et al*., 2000; Feil *et al*., 2003), making the NO-sGC-cGMP signalling one of the most important vasoprotective signalling pathways.

One of the crucial prerequisites of the NO-mediated sGC activation is the presence of the reduced haem moiety. Its oxidation or loss renders the enzyme insensitive to NO. Oxidative stress, a hallmark of many cardiovascular diseases, impairs the NO-cGMP signalling (Melichar *et al*., 2004). Proposed mechanisms include direct chemical scavenging of NO by reactive oxygen species (ROS) such as O_2^-, resulting in a reduced bioavailability of NO and, in parallel, the formation of the strong oxidant peroxynitrite ($ONOO^-$). In turn, this reactive intermediate is able to further inhibit NO signalling by oxidizing the sGC prosthetic haem group to its NO-insensitive Fe^{3+} state (Gladwin, 2006; Stasch *et al*., 2006; Chirkov and Horowitz, 2007). In addition to this acute inactivation of sGC, oxidation of the haem group facilitates the degradation of the enzyme (Stasch *et al*., 2006; Meurer *et al*., 2007). Oxidation-induced impairment of protective NO-cGMP signalling is likely to contribute to endothelial dysfunction in different vascular diseases such as arterial hypertension, atherosclerosis, heart failure and erectile dysfunction (Evgenov *et al*., 2006; Kemp-Harper and Feil, 2008).

For more than a century, provision of NO, as via NO-releasing organic nitrates, has been a major therapeutical approach for the treatment of cardiovascular diseases. However, this class of drugs suffers from several drawbacks including the development of tolerance and, unlike endogenous NO, the lack of any antithrombotic effect on platelets. Moreover, blood vessels suffering from oxidative stress conditions become increasingly unresponsive to NO, a situation that is further aggravated by the fact that organic nitrates increase oxidative stress and have been shown to directly oxidize sGC (Artz *et al*., 2002; Munzel *et al*., 2005; 2007).

As an alternative therapeutic approach, two structurally distinct classes of NO-independent, sGC-activating compounds have been discovered, with the potential to overcome some if not all of the above-mentioned shortcomings (Evgenov *et al*., 2006). Haem-dependent sGC stimulators, including 5-cyclopropyl-2-[1-(2-fluoro-benzyl)-1*H*-pyrazolo [3,4-*b*]pyridin-3-yl]-pyrimidin-4-ylamine (BAY 41-2272), show a strong synergism with NO but lose their ability to stimulate sGC once the prosthetic haem group is oxidized or lost (Stasch *et al*., 2001). In contrast, haem-independent sGC activators (e.g. BAY 58-2667) activate the NO-insensitive oxidized/haem-free form of the enzyme (Stasch *et al*., 2002a) via binding at the enzyme's haem pocket (Schmidt *et al*., 2004; Roy *et al*., 2008). Although this mechanism of binding is generally accepted it is still unclear whether BAY 58-2667 is able to actively compete with the weakly bound haem moiety or if the compound binds solely to the haem-free sGC after the enzyme has lost its oxidized prosthetic group as suggested very recently by Roy *et al*.

By activating sGC and thereby increasing the amount of released cGMP, BAY 58-2667 has different vascular effects. BAY 58-2667 lowers systemic blood pressure, has beneficial effects in hypertension-induced cardiac hypertrophy and inhibits platelet aggregation. In experimental pulmonary hypertension, treatment with BAY 58-2667 leads to a reduction of ventricular systolic pressure and selective pulmonary vasodilatation. Furthermore, BAY 58-2667 decreases the load on the heart and increases cardiac output as well as renal blood flow in experimental congestive heart failure. Preload- and afterload-reducing effects of BAY 58-2667 have been observed in a phase I clinical study and in a phase II clinical trial with patients suffering from acute decompensated heart failure (see Evgenov *et al*., 2006; Schmidt *et al*., 2009).

In addition to its activating effect, BAY 58-2667 is able to rescue the oxidation-impaired sGC from enhanced ubiquitin-mediated degradation, thus accumulating its receptor in a positive feedback loop (Stasch *et al*., 2006; Meurer *et al*., 2007). Compounds mimicking the porphyrinic structure of haem, for example zinc-protoporphyrin IX (Zn-PPIX) and BAY 58-2667, protect sGC from oxidation-induced degradation (Stasch *et al*., 2006). With respect to the structurally unrelated sGC activator, 5-chloro-2-(5-chloro-thiophene-2-sulphonylamino-*N*-(4-(morpholine-4-sulphonyl)-phenyl)-benzamide sodium salt (HMR 1766), competition with different porphyrins suggests an interaction with the enzyme's haem pocket, as shown for BAY 58-2667 (Schindler *et al*., 2006; Stasch *et al*., 2006). This raises the possibility that protection against oxidation-induced degradation is a general feature of haem-independent sGC activators. To test this hypothesis and to further substantiate the mechanisms leading to the observed stabilization of sGC protein levels, we investigated the effects of both compounds and the high affinity metallo-porphyrin, Zn-PPIX, under normal and haem-oxidizing conditions. As the sGC stimulator BAY 41-2272 does not bind to the haem pocket, a stabilizing effect on sGC protein levels was not anticipated, making this compound suitable as negative control.

Experiments were conducted by using two cell models, primary porcine endothelial cells (ECs) and Chinese hamster ovary (CHO) cells expressing wild-type (WT) sGC or the constitutive haem-free sGC mutants β_1H105F and β_1Y135A/R139A. Our findings suggest that BAY 58-2667-like compounds have a unique structural ability to reassemble the spatial structure of the haem moiety within sGC and that this feature allows to prevent sGC degradation in a hitherto not reported drug-induced positive feedback loop on the expression level of its therapeutic target protein.

Methods

Cell culture

Primary ECs were obtained from fresh porcine aortae by collagenase detachment as previously described (Stasch *et al*., 2002b). Briefly, aortae were freed from surrounding tissue, cut open and mounted on a framework with the intima facing upwards. An amount of 20 mL sterile 0.14% collagenase

solution (Biochrom AG, Berlin, Germany) was poured onto the aorta's luminal surface, and the aorta was incubated for 15 min. ECs were scraped from the tissue and cultured until confluent.

cGMP reporter cells were generated and cultured as previously described (Schmidt *et al.*, 2004; Wunder *et al.*, 2005). Briefly, the cGMP reporter cells consist of CHO cells stably transfected with the cGMP-gated Ca^{2+}-channel CNG2 and aequorin, which translates increasing levels of intracellular Ca^{2+} into bioluminescence. In addition, these cells have been stably transfected with WT sGC (α_1 and β_1 subunits of the rat lung enzyme), α_1/β_1H105F sGC or α_1/β_1Y135A/R139A sGC (Becker *et al.*, 1999; Schmidt *et al.*, 2004).

Western blotting

Cells were seeded in six-well plates, grown until confluent and subsequently incubated with 10 μmol·L^{-1} 1H-(1,2,4)-oxadiazolo[4,3-*a*]quinoxalin-1-one (ODQ) alone or combined with 0.01–10 μmol·L^{-1} BAY 58-2667, 10 μmol·L^{-1} BAY 41-2272, 5 μmol·L^{-1} Zn-PPIX or 10 μmol·L^{-1} HMR 1766 respectively. After 24 h, cells were harvested and lysed, and protein was extracted as described earlier (Rothkegel *et al.*, 2007). An amount of 15–30 μg of total protein was separated by SDS-PAGE and transferred to nitrocellulose membrane. The individual sGC subunits were detected by using polyclonal antibodies directed against specific epitopes of the α_1 subunit (Sigma, Steinheim, Germany) and the β_1 subunit (Cayman Chemical Company, Ann Arbor, MI, USA). Actin was used as loading control by using commercially available antibodies (Sigma). Detection was performed by the ECL method (Amersham/GE Healthcare, Buckinghamshire, UK). Protein levels were determined by densitometric analysis of the specific protein bands (GS-800 Calibrated Densitometer, Quantity One Analysis Software, BioRad, Munich, Germany). Values were normalized to the respective control of sGC, which was set to 100% as well as to the respective actin ratio. Data shown in Figures 3 and 8 were obtained in independent sets of experiments using different batches of cells.

sGC activity assays

cGMP concentrations of ECs were determined by a commercially available radio-immuno assay kit (IBL, Hamburg, Germany; Stasch *et al.*, 2002a; Schmidt, 2009). In the cGMP reporter cell line, sGC activity was determined 48 h after seeding (Schmidt *et al.*, 2004; Wunder *et al.*, 2005). Briefly, cells were incubated with increasing concentrations of the respective test substances for 10 min. Subsequently, 10 mmol·L^{-1} $CaCl_2$ was added, and the resulting bioluminescence directly correlated with intracellular cGMP concentrations (Wunder *et al.*, 2005). Values were expressed as relative light units (RLUs).

The activity of haem-free recombinant rat sGC was assayed via the formation of [^{32}P]-cGMP from [α-^{32}P]-GTP in the presence of Mg^{2+}(Hoenicka *et al.*, 1999; Schmidt, 2009). Removal of the haem group was achieved by adding 2% Tween-20 to the reaction buffer, as previously described (Foerster *et al.*, 1996; Schmidt *et al.*, 2003). sGC was incubated with 100 nmol·L^{-1} BAY 58-2667 or 100 μmol·L^{-1} HMR 1766, which resulted in similar fold stimulation. These fixed concentrations of sGC activators were combined with increasing concentrations of Zn-PPIX.

Receptor binding assay

Homologous and heterologous competition binding studies were performed by using a receptor binding assay, as described previously (Schmidt *et al.*, 2003). An amount of 1 μg sGC was incubated with 100 nmol·L^{-1} ^{3}H-BAY 58-2667 and increasing concentrations of unlabelled BAY 58-2667, HMR 1766 or Zn-PPIX respectively. Free and bound radioligands were separated via 96-well filter plates coated with polyvinylpyrrolidone. Bound radioactivity was determined by scintillation counting. Non-specific binding was measured by the addition of a 1000-fold excess of unlabelled BAY 58-2667 and subtracted from total binding in every individual assay.

Statistics

Data are presented as means ± standard error of the mean (SEM). GraphPad Prism software version 4.02 (GraphPad Software Inc., San Diego, CA, USA) was used for curve fitting and calculation of EC_{50} or IC_{50} values. Ninety-five per cent confidence intervals of EC_{50} and half maximal inhibitory concentration (IC_{50}) values are given in parentheses. Statistical comparisons were performed by using the paired Student's *t*-test.

Materials

BAY 58-2667, BAY 41-2272 and HMR 1766 were synthesized as described (Figure 1; Straub *et al.*, 2001; Stasch *et al.*, 2002b; Schindler *et al.*, 2006). Tritium labelling of BAY 58-2667 was performed as described (Shu and Heys, 2000). ODQ was purchased from Tocris Bioscience (Avonmouth, UK); Zn-PPIX (zinc-3,18-divinyl-2,7,13,17-tetramethylporphine-8,12-dipropionic acid), from Sigma. All other chemicals were of analytical grade and obtained from Sigma.

Results

Inhibition of sGC activity by Zn-PPIX

To validate the hypothesis that HMR 1766 interacts with the sGC haem pocket as shown for BAY 58-2667, purified recombinant haem-free sGC was incubated with concentrations of BAY 58-2667 or HMR 1766 that activated the enzyme to a similar extent (Figure 2A). BAY 58-2667 (100 nmol·L^{-1}) activated the enzyme 69.5-fold (reflecting a specific activity of 10.2 μmol cGMP·mg^{-1}·min^{-1}). At a concentration of 100 μM, HMR 1766 induced a comparable 72.9-fold activation (to 13.6 μmol cGMP·mg^{-1}·min^{-1}). The addition of increasing concentrations of Zn-PPIX resulted in an inhibition of activated sGC with IC_{50} values of either 4.8 (2.2–10.2) nmol·L^{-1} (for BAY 58-2667-activated sGC) or 2.2 (0.9–5.3) nmol·L^{-1} (for HMR 1766-activated sGC), indicating that both compounds interacted with the sGC haem pocket.

Competition binding of BAY 58-2667 and HMR 1766

A receptor binding assay using ^{3}H-labelled BAY 58-2667 was used to further investigate whether BAY 58-2667 and HMR

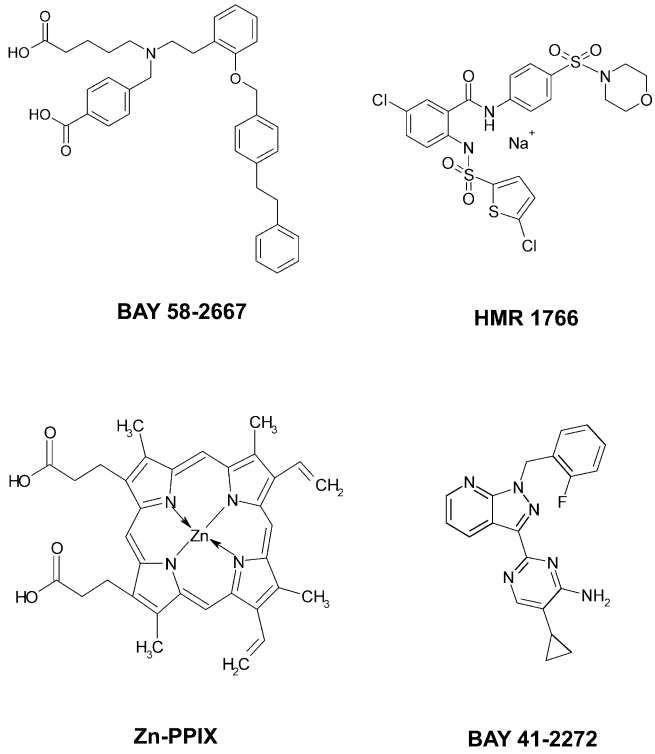

Figure 1 Chemical structures of the sGC activators BAY 58-2667 and HMR 1766, the sGC stimulator BAY 41-2272 and the haem pocket antagonist zinc protoporphyrin IX (Zn-PPIX). BAY 41-2272, 5-cyclopropyl-2-[1-(2-fluoro-benzyl)-1*H*-pyrazolo[3,4-*b*]pyridin-3-yl]-pyrimidin-4-ylamine; BAY 58-2667, 4-[((4-carboxybutyl){2-[(4-phenethylbenzyl)oxy]phenethyl}amino)methyl[benzoic]acid; HMR 1766, 5-chloro-2-(5-chloro-thiophene-2-sulphonylamino-*N*-(4-(morpholine-4-sulphonyl)-phenyl)-benzamide sodium salt; sGC, soluble guanylyl cyclase; Zn-PPIX, zinc-protoporphyrin IX.

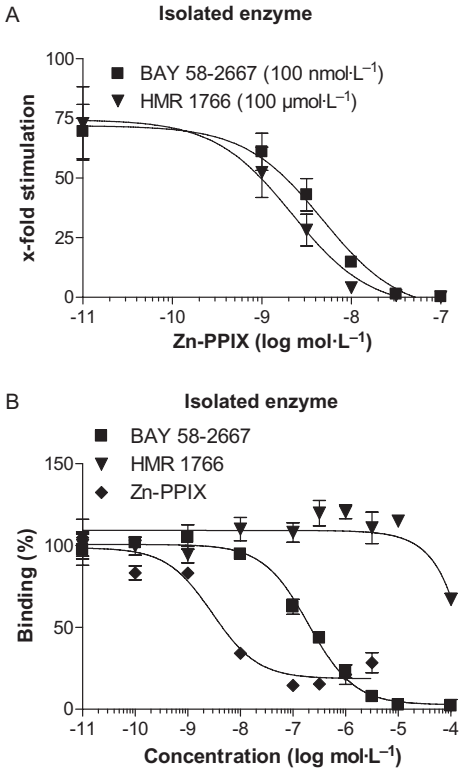

Figure 2 (A) Inhibition of BAY 58-2667 or HMR 1766-induced sGC activation by Zn-PPIX. Activity was measured by formation of [^{32}P]-cGMP from [α-^{32}P]-GTP. Isolated sGC was incubated with 100 nmol·L^{-1} BAY 58-2667 or 100 µmol·L^{-1} HMR 1766 and increasing concentrations of Zn-PPIX. Data are shown as means ± SEM from five independent experiments performed in duplicate. (B) Comparison of the competition binding of BAY 58-2667, HMR 1766 and Zn-PPIX. Displacement of 100 nmol·L^{-1} ^{3}H-BAY 58-2667 was studied in a receptor binding assay. BAY 58-2667 showed an average non-specific binding of 717 dpm and maximal values of 4338 dpm. HMR 1766 had an average non-specific binding of 616 dpm and maximal values of 4149 dpm. Data are means ± SEM from three to five independent experiments performed in duplicate. BAY 58-2667, 4-[((4-carboxybutyl){2-[(4-phenethylbenzyl)oxy]phenethyl}amino) methyl[benzoic]acid; HMR 1766, 5-chloro-2-(5-chloro-thiophene-2-sulphonylamino-*N*-(4-(morpholine-4-sulphonyl)-phenyl)-benzamide sodium salt; SEM, standard error of the mean; sGC, soluble guanylyl cyclase; Zn-PPIX, zinc-protoporphyrin IX.

1766 directly compete for the same binding site (Figure 2B). Radioactively labelled BAY 58-2667 was incubated with increasing concentration of unlabelled BAY 58-2667, HMR 1766 or Zn-PPIX. Unlabelled BAY 58-2667 displayed an IC_{50} of 200 (140.1–281.4) nmol·L^{-1}. Based on a binding constant (K_D) of 13.4 nmol·L^{-1} (Schmidt *et al.*, 2003), a K_i of 23 nmol·L^{-1} was estimated. Zn-PPIX displaced ^{3}H-BAY 58-2667 with an IC_{50} of 2.9 (1.3–6.3) nmol·L^{-1}. HMR 1766 competed with ^{3}H-BAY 58-2667 only at very high concentrations of ≥10 µmol·L^{-1}.

Levels of sGC protein in cGMP reporter cells under normal and haem-oxidizing conditions

sGC degradation was induced by incubating cells for 24 h with the sGC inhibitor ODQ (Garthwaite *et al.*, 1995; Olesen *et al.*, 1998; Zhao *et al.*, 2000). Under these conditions, sGC protein levels decreased by 59.7 and 35.6% for the α_1 and β_1 sGC subunit respectively (Figure 3). Control experiments in which cells were incubated with ODQ for only 30 min did not induce any significant changes in sGC protein levels (data not shown). Co-incubation with the sGC activator, BAY 58-2667, prevented the ODQ-induced decrease in sGC protein levels for both subunits, concentration dependently with a minimal effective concentration of 10 nmol·L^{-1} (Figure 3, Table 1). sGC protein levels of cells treated with BAY 58-2667 alone (i.e. without haem oxidation by ODQ) remained constant (Figure 3, Table 1). In contrast, the structurally unrelated sGC activator HMR 1766 showed no alteration in sGC protein levels, neither in control nor ODQ-treated reporter cells (Figure 3). Conversely, exposure of cGMP reporter cells to the competitive haem pocket antagonist Zn-PPIX led to a slight reduction of sGC protein levels. However, upon haem oxidation, sGC protein levels in Zn-PPIX-treated cells were higher than in cells exposed to ODQ alone. As expected, the sGC stimulator BAY 41-2272 had no effect on sGC protein levels, neither under control nor under haem-oxidizing conditions.

Effects of haem oxidation on sGC protein levels in ECs

Long-term (24 h, Figure 4) but not short-term (30 min, data not shown) incubation with ODQ decreased sGC α_1 and β_1 levels by 57.8 and 46.0% respectively. Unlike the results from

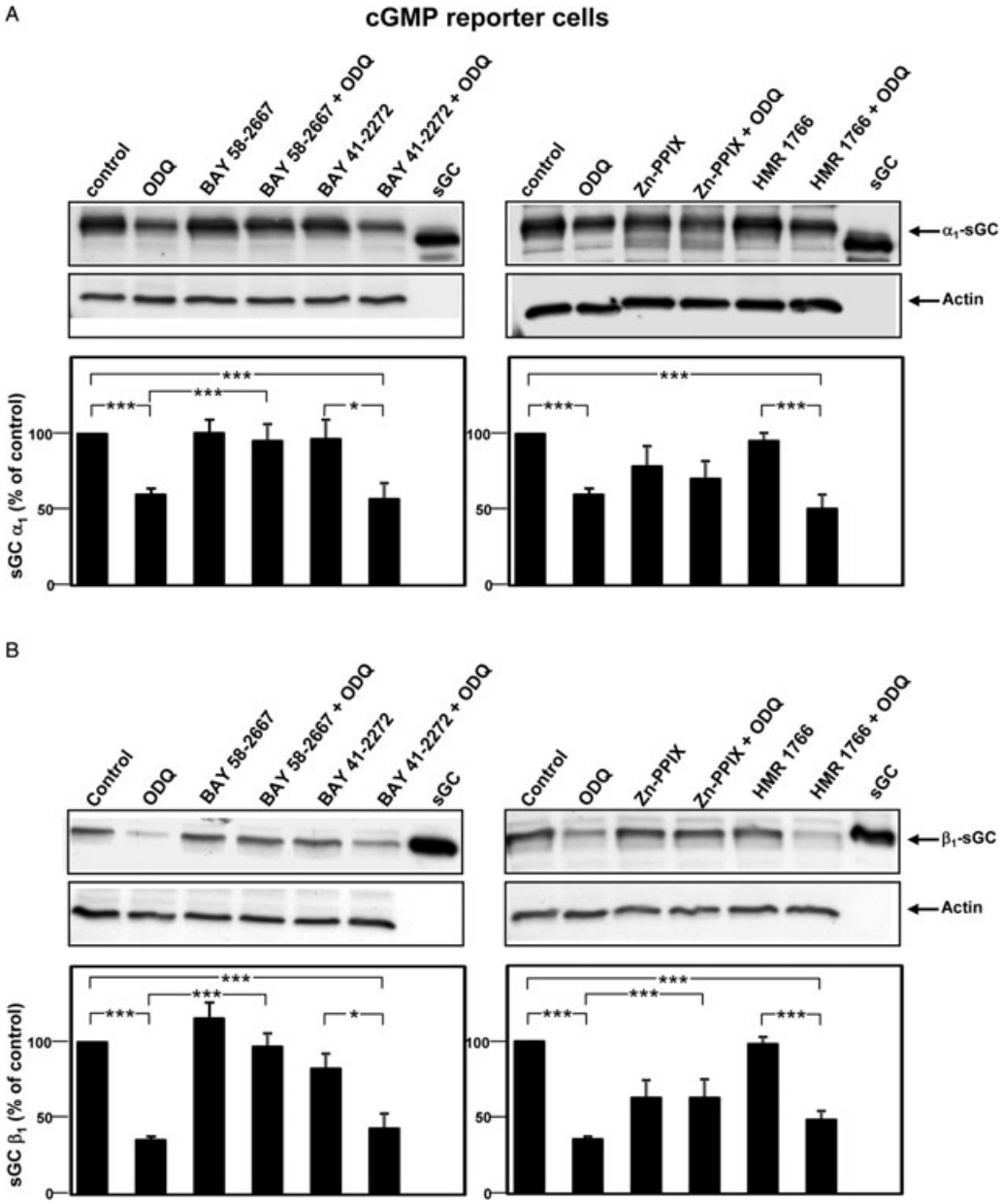

Figure 3 Effects of 10 μmol·L^{-1} BAY 58-2667, 10 μmol·L^{-1} BAY 41-2272, 10 μmol·L^{-1} HMR 1766 and 5 μmol·L^{-1} Zn-PPIX on sGC protein levels under normal and haem-oxidizing conditions in cGMP reporter cells. Haem oxidation was achieved by pre-incubating cells with 10 μmol·L^{-1} ODQ for 24 h. (A) Representative Western blots of α_1 sGC and actin as a loading control (upper panel). Purified rat sGC was used as control. α_1 sGC protein levels as determined by densitometric measurement are shown in the lower panel. (B) Representative Western blots of β_1 sGC and actin as a loading control (upper panel). Purified rat sGC was used as control. β_1 sGC protein levels as determined by densitometric measurement are shown in the lower panel. sGC protein levels are expressed as percentage of the respective control which was set as 100% (means ± SEM of 5–32 independent experiments). *P < 0.05, **P < 0.01, ***P < 0.005: Student's t-test. BAY 41-2272, 5-cyclopropyl-2-[1-(2-fluoro-benzyl)-1*H*-pyrazolo[3,4-*b*]pyridin-3-yl]-pyrimidin-4-ylamine; BAY 58-2667,4-[((4-carboxybutyl){2-[(4-phenethylbenzyl)oxy] phenethyl}amino)methyl [benzoic] acid; HMR, 5-chloro-2-(5-chloro-thiophene-2-sulphonylamino-*N*-(4-(morpholine-4-sulphonyl)-phenyl)-benzamide sodium salt; ODQ, 1H-(1,2,4)-oxadiazolo[4,3-*a*]quinoxalin-1-one; sGC, soluble guanylyl cyclase; Zn-PPIX, zinc-protoporphyrin IX.

the cGMP reporter cell line, BAY 58-2667 induced a concentration-dependent increase of the β_1 subunit beyond control even in the absence of ODQ, whereas α_1 protein levels remained unchanged (Figure 4, Table 2). Similar results were obtained in ODQ-treated ECs, where BAY 58-2667 increased β_1 protein levels beyond the amounts observed in untreated controls, whereas α_1 levels were stabilized at the level of untreated controls (Table 2, Figure 4). Similar to BAY 58-2667, exposure of ECs to Zn-PPIX resulted in increased sGC β_1 protein levels both under control conditions and upon haem oxidation beyond the levels of the respective controls. And even upon haem oxidation, α_1 protein levels of Zn-PPIX-treated cells were higher than in cells treated with only ODQ. Conversely, neither the NO-independent sGC agonists BAY 41-2272 nor HMR 1766 relevantly increased the levels of either sGC subunit in ECs under any of the tested conditions.

Table 1 Effects of increasing concentrations of BAY 58-2667 on sGC protein levels under normal and haem-oxidizing conditions in cGMP reporter cells stably transfected with WT sGC

cGMP reporter cells	*α_1 sGC*		*β_1 sGC*	
BAY 58-2667	*ODQ (10 μmol·L^{-1})*		*ODQ (10 μmol·L^{-1})*	
[μmol·L^{-1}]	–	+	–	+
–	100	60 ± 4***	100	36 ± 3***
0.01	88 ± 7***††	65 ± 5***#	79 ± 5***†††	38 ± 10***††
0.1	93 ± 15†††	76 ± 8***	73 ± 10***†††	63 ± 13***†††
1	94 ± 8***†††	94 ± 6***†††	92 ± 25†††	88 ± 33†††
10	100 ± 9†††	95 ± 11†††	116 ± 10†††	97 ± 9†††

Values are expressed as % control (no ODQ or BAY 58-2667) and are means ± SEM of 3–32 independent experiments.
***$P < 0.005$: Student's *t*-test (indicated sample vs. control); ††$P < 0.01$, †††$P < 0.005$: Student's *t*-test (indicated sample vs. ODQ treated control); #$P < 0.05$: Student's *t*-test (substance treatment vs. substance plus ODQ treatment).
BAY 58-2667, 4-[((4-carboxybutyl){2-[(4-phenethylbenzyl)oxy]phenethyl}amino)methyl[benzoic]acid; cGMP, cyclic guanosine monophosphate; ODQ, 1H-(1,2,4)-oxadiazolo[4,3-*a*]quinoxalin-1-one; SEM, standard error of the mean; sGC, soluble guanylyl cyclase; WT, wild type.

sGC activity in cGMP reporter cells

For the effects of the haem oxidant ODQ in cGMP reporter cells to be examined, two approaches were tested, namely a long-term incubation (24 h) to reduce sGC protein levels, as well as a short-term incubation (10 min) with ODQ to oxidize the sGC haem group and inhibit activity without affecting the enzyme's protein level. Moreover, long-term treated cells were additionally short-term treated to test whether the BAY 58-2667-induced activity of residual sGC can be further enhanced by acute oxidation. Figure 5 summarizes the results of these protocols on the subsequent sGC activation by BAY 58-2667, BAY 41-2272, Zn-PPIX or HMR 1766 respectively.

BAY 58-2667 (30 μmol·L^{-1}) activated sGC up to 73-fold with an EC_{50} value of 23.3 (13.1–41.3) nmol·L^{-1}. Short-term exposure to ODQ enhanced sGC activation to 129-fold with a decrease in the EC_{50} to 9.1 (4.6–17.9) nmol·L^{-1} (Figure 5A). Long-term incubation with ODQ resulted in only 78-fold sGC activation by BAY 58-2667 with a slightly higher EC_{50} of 15.1 (9.7–23.6) nmol·L^{-1}. When long-term incubated cells were additionally exposed to a second short-term application of ODQ, sensitivity to BAY 58-2667 was similar [EC_{50} 9.9 (4.0–24.5) nmol·L^{-1}, 82-fold activation].

BAY 41-2272 (30 μmol·L^{-1}) stimulated sGC up to 115-fold with an EC_{50} of 596 (364.7–973.5) nmol·L^{-1}. Short-term treatment with ODQ (10 min) reduced sGC stimulation to 23-fold with a corresponding increase in the EC_{50} to 831 (414.8–1665) nmol·L^{-1} (Figure 5B). Long-term haem oxidation resulted in a further decrease in sGC activity [maximal stimulation 17-fold, EC_{50} value of 580 (352.4–953.7) nmol·L^{-1}]. Additional acute oxidation of long-term treated cells led to a similar EC_{50} value of 607 (197.1–1868) nmol·L^{-1} for BAY 41-2272-induced sGC activity as in cells that has only long-term treatment. As expected for a full antagonist, Zn-PPIX had no effect on sGC activity, neither under haem-oxidizing nor under control conditions (Figure 5D).

Treatment with increasing concentrations of HMR 1766 resulted in an up to 557-fold activation with an EC_{50} value of 8.8 (6.6–11.8) μmol·L^{-1} (Figure 5C). Surprisingly, acute ODQ did not increase HMR 1766-induced activation, and long-term ODQ even slightly diminished HMR 1766-induced sGC activation (Figure 5D), which was also not changed by additional acute ODQ exposure [EC_{50} 13.5 (6.5–27.9) μmol·L^{-1}].

sGC activity in ECs

In ECs, sGC activity was determined by measuring cGMP accumulation via radio-immuno assay upon incubating cells with increasing concentrations of BAY 58-2667, Zn-PPIX, HMR 1766 or BAY 41-2272 under normal or haem-oxidizing conditions (Figure 6) respectively. BAY 58-2667 showed a flat concentration response curve, and the maximal activation was only eightfold with an EC_{50} value of 0.3 (0.03–2.3) μmol·L^{-1}. Haem oxidation potentiated BAY 58-2667-induced sGC activation up to 134-fold at the highest applied concentration of 10 μmol·L^{-1}, with an EC_{50} value of 0.2 (0.05–0.7) μmol·L^{-1} (Figure 6A).

BAY 41-2272 increased cGMP levels up to 123-fold at the highest tested concentration of 10 μmol·L^{-1}, which was reduced to 86-fold after long-term ODQ treatment. The corresponding EC_{50} value was shifted from 737 (326.1–1667) to 946 (411.8–2175) nmol·L^{-1} respectively (Figure 6B). Incubating ECs with HMR 1766 lead to a maximal activation of 20-fold, and this was increased to 749-fold upon haem oxidation. EC_{50} values were 1261 (0.0–28545) and 810 (0.0–419) μmol·L^{-1} respectively (Figure 6C).

Activities of mutant β_1H105F and β_1Y135A/R139A sGCs

The activity of sGC containing either β_1H105F or β_1Y135A/R139A was determined in cGMP reporter cells stably transfected with expression vectors encoding for the respective enzyme mutants. WT, β_1H105F and β_1Y135A/R139A sGCs were incubated with increasing concentrations of BAY 58-2667 or HMR 1766 alone or combination with short-term ODQ treatment (Figure 7). WT sGC was activated up to 17-fold by 30 μmol·L^{-1} BAY 58-2667, with an EC_{50} of 0.2 (0.02–1.1) μmol·L^{-1}, and this activation was increased to 64-fold with an EC_{50} of 2.0 (0.4–9.3) μmol·L^{-1} by addition of 10 μmol·L^{-1} ODQ (Figure 7A). Incubation of these WT sGC expressing cells with HMR 1766 resulted in a maximal activation of 230-fold by 30 μmol·L^{-1} HMR 1766, with an EC_{50} of

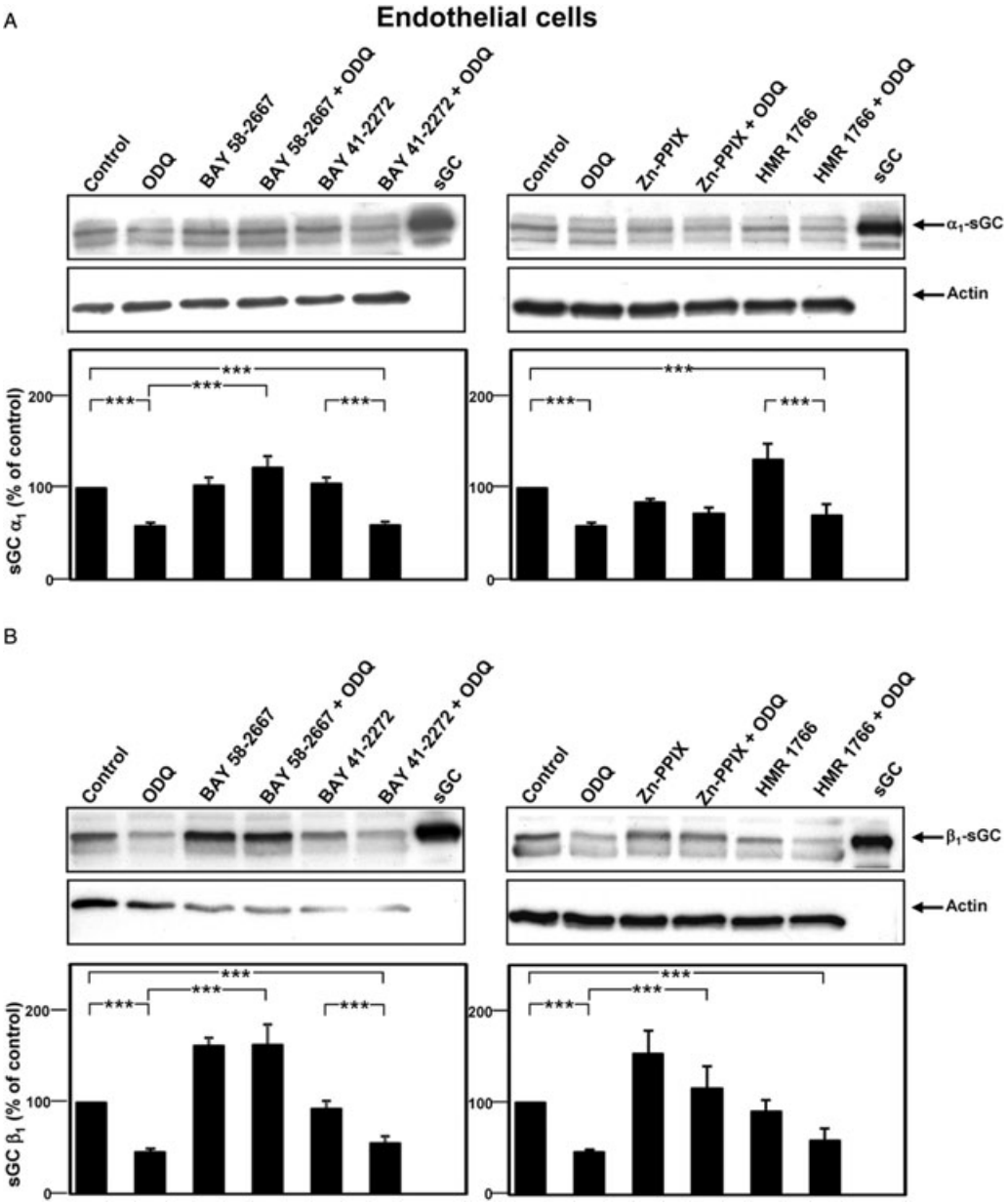

Figure 4 Effects of 10 μmol·L^{-1} BAY 58-2667, 10 μmol·L^{-1} BAY 41-2272, 10 μmol·L^{-1} HMR 1766 and 5 μmol·L^{-1} Zn-PPIX on sGC protein levels under normal and haem-oxidizing conditions in endothelial cells. Haem oxidation was achieved by incubation of the cells with 10 μmol·L^{-1} ODQ for 24 h. (A) Representative Western blots of α_1 sGC and actin as a loading control (upper panel). Purified rat sGC was used as control. α_1 sGC protein levels as determined by densitometric measurement are shown in the lower panel. (B) Representative Western blots of β_1 sGC and actin as a loading control (upper panel). Purified rat sGC was used as control. β_1 sGC protein levels as determined by densitometric measurement are shown in the lower panel. sGC protein levels are expressed as percentage of the respective control which was set as 100% (means ± SEM of 5–29 independent experiments). *P < 0.05, **P < 0.01, ***P < 0.005: Student's *t*-test. BAY 41-2272, 5-cyclopropyl-2-[1-(2-fluoro-benzyl)-1*H*-pyrazolo[3,4-*b*]pyridin-3-yl]-pyrimidin-4-ylamine; BAY 58-2667, 4-[((4-carboxybutyl){2-[(4-phenethylbenzyl)oxy] phenethyl}amino)methyl[benzoic]acid; HMR 1766, 5-chloro-2-(5-chloro-thiophene-2-sulphonylamino-*N*-(4-(morpholine-4-sulphonyl)-phenyl)-benzamide sodium salt; ODQ, 1H-(1,2,4)-oxadiazolo[4,3-*a*]quinoxalin-1-one; sGC, soluble guanylyl cyclase; Zn-PPIX, zinc-protoporphyrin IX.

4.2 (1.6–10.8) μmol·L^{-1}. Co-incubation with ODQ increased the activation to 328-fold with an EC_{50} of 1.5 (0.7–3.0) μmol·L^{-1} (Figure 7B).

Incubation of cGMP reporter cells expressing the haem-free sGC mutant β_1H105F with BAY 58-2667 resulted in a maximal activation of 45-fold, which was only slightly increased to 55-fold by addition of ODQ [determined EC_{50} were 11.6 (3.2–41.8) nmol·L^{-1} and 7.6 (1.8–31.2) nmol·L^{-1} respectively (Figure 7C)]. The highest concentration of HMR 1766 (30 μmol·L^{-1}) activated β_1H105F cells up to 95-fold [EC_{50} 0.8 (0.5–1.1) μmol·L^{-1}] and 100-fold [EC_{50} 0.8 (0.4–1.8) μmol·L^{-1}] by the addition of ODQ (Figure 7D). The double mutant β_1Y135A/R139A was activated neither by BAY 58-2667 nor by BAY 58-2667 in combination with ODQ (Figure 7E) and only

Table 2 Effects of increasing concentrations of BAY 58-2667 on sGC protein levels under normal and haem-oxidizing conditions in endothelial cells

ECs	α_1 *sGC*		β_1 *sGC*	
BAY 58-2667	*ODQ (10 μmol·L^{-1})*		*ODQ (10 μmol·L^{-1})*	
[μmol·L^{-1}]	–	+	–	+
–	100	58 ± 4***	100	46 ± 3***
0.01	108 ± 3***†††	60 ± 8***$^{\#\#\#}$	106 ± 5***†††	53 ± 5***$^{\#\#\#}$
0.1	99 ± 11††	64 ± 15***	111 ± 11**†††	74 ± 9***$^{\dagger\dagger\dagger\#}$
1	100 ± 9†††	92 ± 9**††	138 ± 13***†††	129 ± 17***†††
10	103 ± 9†††	122 ± 12***†††	162 ± 24***†††	163 ± 22***†††

Values are expressed as % control (no ODQ or BAY 58-2667) and are means ± SEM of 4–29 independent experiments.
**P < 0.01, ***P < 0.005: Student's t-test (indicated sample vs. control); $^{\dagger\dagger}P$ < 0.01, $^{\dagger\dagger\dagger}P$ < 0.005: Student's t-test (indicated sample vs. ODQ treated control); $^{\#}P$ < 0.05, $^{\#\#\#}P$ < 0.005: Student's t-test (substance treatment vs. substance plus ODQ treatment).
BAY 58-2667, 4-[((4-carboxybutyl){2-[(4-phenethylbenzyl)oxy]phenethyl}amino)methyl[benzoic]acid; cGMP, cyclic guanosine monophosphate; EC, endothelial cell; ODQ, 1H-(1,2,4)-oxadiazolo[4,3-*a*]quinoxalin-1-one; SEM, standard error of the mean; sGC, soluble guanylyl cyclase.

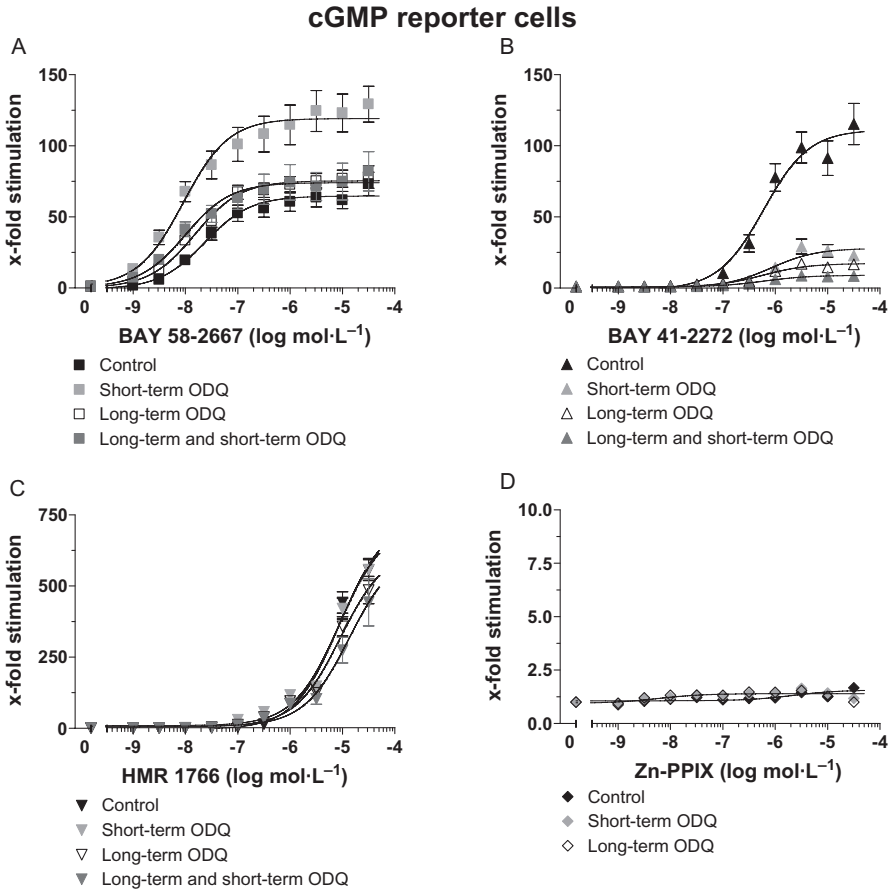

Figure 5 Concentration response curves of wildtype sGC in cGMP reporter cells incubated with increasing concentrations of BAY 58-2667 (A), BAY 41-2272 (B), HMR 1766 (C) or Zn-PPIX (D) alone, in combination with 10 μmol·L^{-1} ODQ for 10 min, after 24 h pretreatment with 10 μmol·L^{-1} ODQ and pretreatment with additional treatment with 10 μmol·L^{-1} ODQ for 10 min. Data are means ± SEM from 7–19 independent experiments performed in quadruplicate. sGC activation is represented as *x*-fold stimulation compared with non-stimulated control. Following basal activities were measured: (A) 10 min BAY 58-2667 1091 RLUs; 10 min BAY 58-2667 + ODQ 867 RLUs; 24 h ODQ/10 min BAY 58-2667 1441 RLUs; 24 h ODQ/10 min BAY 58-2667 + ODQ 866 RLUs. (B) 10 min BAY 41-2272 980 RLUs; 10 min BAY 41-2272 + ODQ 1800 RLUs; 24 h ODQ/10 min BAY 41-2272 1069 RLUs; 24 h ODQ/10 min BAY 41-2272 + ODQ 584 RLUs. (C) 10 min HMR 1766 1528 RLUs; 10 min HMR 1766 + ODQ 1380 RLUs; 24 h ODQ/10 min HMR 1766 1411 RLUs; 24 h ODQ/10 min HMR 1766 + ODQ 856 RLUs. (D) 10 min Zn-PPIX 1130 RLUs; 10 min Zn-PPIX + ODQ 1131 RLUs; 24 h ODQ/10 min Zn-PPIX 1750 RLUs. BAY 41-2272, 5-cyclopropyl-2-[1-(2-fluoro-benzyl)-1*H*-pyrazolo[3,4-*b*]pyridin-3-yl]-pyrimidin-4-ylamine; BAY 58-2667, 4-[((4-carboxybutyl){2-[(4-phenethylbenzyl)oxy]phenethyl}amino)methyl[benzoic]acid; cGMP, cyclic guanosine monophosphate; HMR 1766, 5-chloro-2-(5-chloro-thiophene-2-sulphonylamino-*N*-(4-(morpholine-4-sulphonyl)-phenyl)-benzamide sodium salt; ODQ, 1H-(1,2,4)-oxadiazolo[4,3-*a*]quinoxalin-1-one; RLU, relative light unit; SEM, standard error of the mean; sGC, soluble guanylyl cyclase; Zn-PPIX, zinc-protoporphyrin IX.

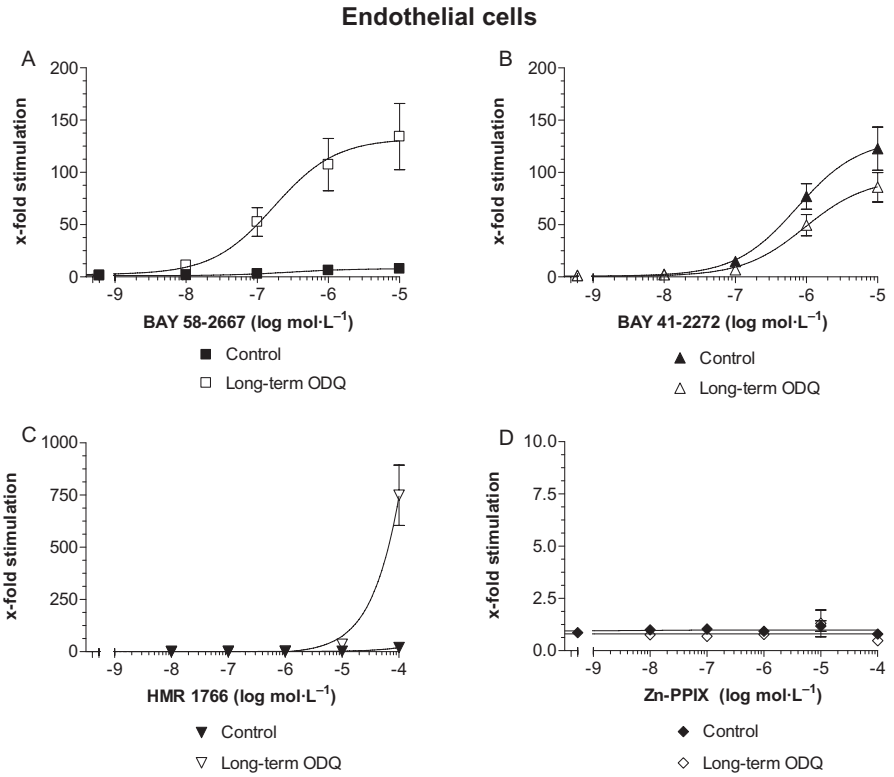

Figure 6 Effects of BAY 58-2667, BAY 41-2272, HMR 1766 and Zn-PPIX on sGC activity under normal and haem-oxidizing conditions. Endothelial cells were treated with different concentrations of BAY 58-2667 (A), HMR 1766 (C), Zn-PPIX (D) for 30 min or BAY 41-2272 (B) for 15 min with or without 24 h ODQ (10 μmol·L^{-1}) pretreatment. sGC activity was determined by measurement of cGMP accumulation via radio-immunoassay. Data are expressed as *x*-fold stimulation of control (means ± SEM of 8–14 independent experiments). The basal cGMP contents was as follows: (in fmol cGMP per well): (A) BAY 58-2667 409; BAY 58-2667 + ODQ 24 h 19; (B) BAY 41-2272 484; BAY 41-2272 + ODQ 24 h 26; (C) HMR 1766 241; HMR 1766 + ODQ 24 h 16; (D) Zn-PPIX 408; Zn-PPIX + ODQ 24 h 19. BAY 41-2272, 5-cyclopropyl-2-[1-(2-fluoro-benzyl)-1*H*-pyrazolo[3,4-*b*]pyridin-3-yl]-pyrimidin-4-ylamine; BAY 58-2667, 4-[((4-carboxybutyl){2-[(4-phenethylbenzyl) oxy]phenethyl}amino)methyl[benzoic]acid; cGMP, cyclic guanosine monophosphate; HMR 1766, 5-chloro-2-(5-chloro-thiophene-2-sulphonylamino-*N*-(4-(morpholine-4-sulphonyl)-phenyl)-benzamide sodium salt; ODQ, 1H-(1,2,4)-oxadiazolo[4,3-*a*]quinoxalin-1-one; sGC, soluble guanylyl cyclase; Zn-PPIX, zinc-protoporphyrin IX.

slightly activated by HMR 1766 (5-fold) or HMR 1766 and ODQ (6-fold; Figure 7F).

sGC protein levels of β_1H105F sGC and β_1Y135A/R139A sGC

Incubation of the WT sGC expressing cell line with ODQ for 24 h decreased sGC protein levels by 49% (α_1) and 61% (β_1) (Figure 8). BAY 58-2667 was able to rescue sGC α_1 protein to control levels, whereas sGC β_1 was increased beyond control. This observation was not affected by additional ODQ. In the cell line expressing the haem-free mutant β_1H105F, oxidation did not result in reduced protein levels of both subunits. The addition of BAY 58-2667 both under control and haem-oxidizing conditions strongly increased β_1H105F protein levels with weaker effects on α_1 sGC. In cells expressing haem-free β_1Y135A/R139A, sGC protein levels remained unchanged both under control and haem-oxidizing conditions, and BAY 58-2667 had no effect on the protein levels of either subunit (Figure 8).

Discussion

The NO-cGMP pathway plays a key role in the cardiovascular system, and its impairment is associated with different cardiovascular diseases. Recent findings suggest that increased levels of oxidative stress as observed under pathophysiological conditions can lead to oxidation or even loss of the sGC haem group, rendering the enzyme insensitive to NO and prone to ubiquitin-mediated degradation (Stasch *et al.*, 2006; Meurer *et al.*, 2007; Xia *et al.*, 2007). These results might explain at least partially the observed reduction of sGC protein levels in different animal models of cardiovascular diseases (Ruetten *et al.*, 1999; Kagota *et al.*, 2001; Melichar *et al.*, 2004).

Occupation of the sGC haem pocket with high affinity metallo-porphyrins or compounds resembling the spatial structure and charge distribution of haem such as BAY 58-2667 is able to prevent the oxidation-induced degradation of sGC (Stasch *et al.*, 2006; Meurer *et al.*, 2007). In parallel, Schindler *et al.*, (2006) identified another haem-independent sGC activator that, although structurally unrelated, shares some characteristics with BAY 58-2667 such as the activation of haem-free sGC. The first evidence suggested an interaction of HMR 1766 with the haem pocket, as described for metallo-porphyrins and BAY 58-2667. However, whether this compound binds to the sGC haem site and if it is able to prevent the oxidation-induced sGC degradation as shown for Zn-PPIX and BAY 58-2667 have not been investigated yet. To clarify

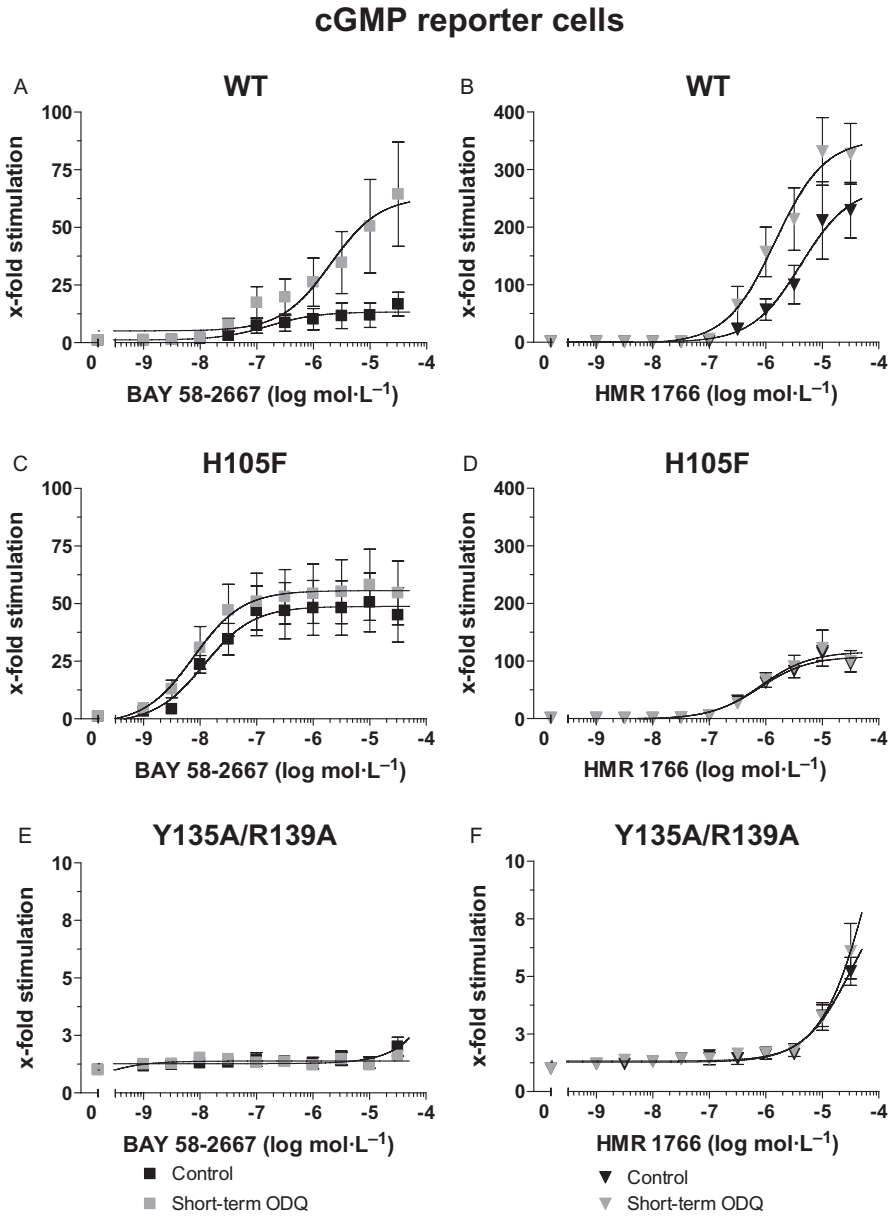

Figure 7 Concentration response curves of cGMP reporter cells stably transfected with WT (A, B) α_1/β_1H105F sGC (C, D) or α_1/β_1Y135A/R139A sGC (E, F) incubated with increasing concentrations of BAY 58-2667 (A, C ,E) or HMR 1766 (B, D, F) alone or in combination with short-term ODQ (10 μmol·L^{-1}) treatment. Data are means ± SEM from four to eight independent experiments performed in duplicate. sGC activation is represented as *x*-fold stimulation compared with non-stimulated control. Following basal activities from which *x*-fold stimulation was calculated were measured: (A) BAY 58-2667 2986 RLUs; BAY 58-2667 + ODQ 10 min 3078 RLUs; (B) HMR 1766 2957 RLUs; HMR 1766 + ODQ 10 min 2654 RLUs; (C) BAY 58-2667 16796 RLUs; BAY 58-2667 + ODQ 10 min 18266 RLUs; (D) HMR 1766 16624 RLUs; HMR 1766 + ODQ 10 min 19589 RLUs; (E) BAY 58-2667 3626 RLUs, BAY 58-2667 + ODQ 10 min 3822 RLUs; (F) HMR 1766 3494 RLUs; HMR 1766 + ODQ 10 min 3498 RLUs. BAY 58-2667, 4-[((4-carboxybutyl){2-[(4-phenethylbenzyl)oxy]phenethyl}amino)methyl[benzoic]acid; cGMP, cyclic guanosine monophosphate; HMR 1766, 5-chloro-2-(5-chloro-thiophene-2-sulphonylamino-*N*-(4-(morpholine-4-sulphonyl)-phenyl)-benzamide sodium salt; ODQ, 1H-(1,2,4)-oxadiazolo[4,3-*a*]quinoxalin-1-one; RLU, relative light unit; sGC, soluble guanylyl cyclase; WT, wildtype.

this open question and to shed light on the general mechanism of sGC stabilization, the present study investigated the molecular requirements of sGC activation and stabilization using the sGC activators, BAY 58-2667 and HMR 1766, and the haem pocket antagonist, Zn-PPIX, on sGC activity and protein levels in two cell systems expressing WT and mutant sGC.

To validate a putative interaction of HMR 1766 with the sGC haem pocket, sGC activity and competition binding assays were performed with BAY 58-2667, HMR 1766 and Zn-PPIX. Activity assays with purified sGC showed unequivocally that the high-affinity metallo-porphyrin is able to inhibit BAY 58-2667 and HMR 1766-induced sGC activation, suggesting an interaction of HMR 1766 with the haem-binding site as shown for BAY 58-2667 (Schmidt *et al*., 2004; Schindler *et al*., 2006; Stasch *et al*., 2006). The competition binding assays performed here support this view, as unlabelled BAY 58-2667 and Zn-PPIX displaced ^{3}H-BAY 58-2667

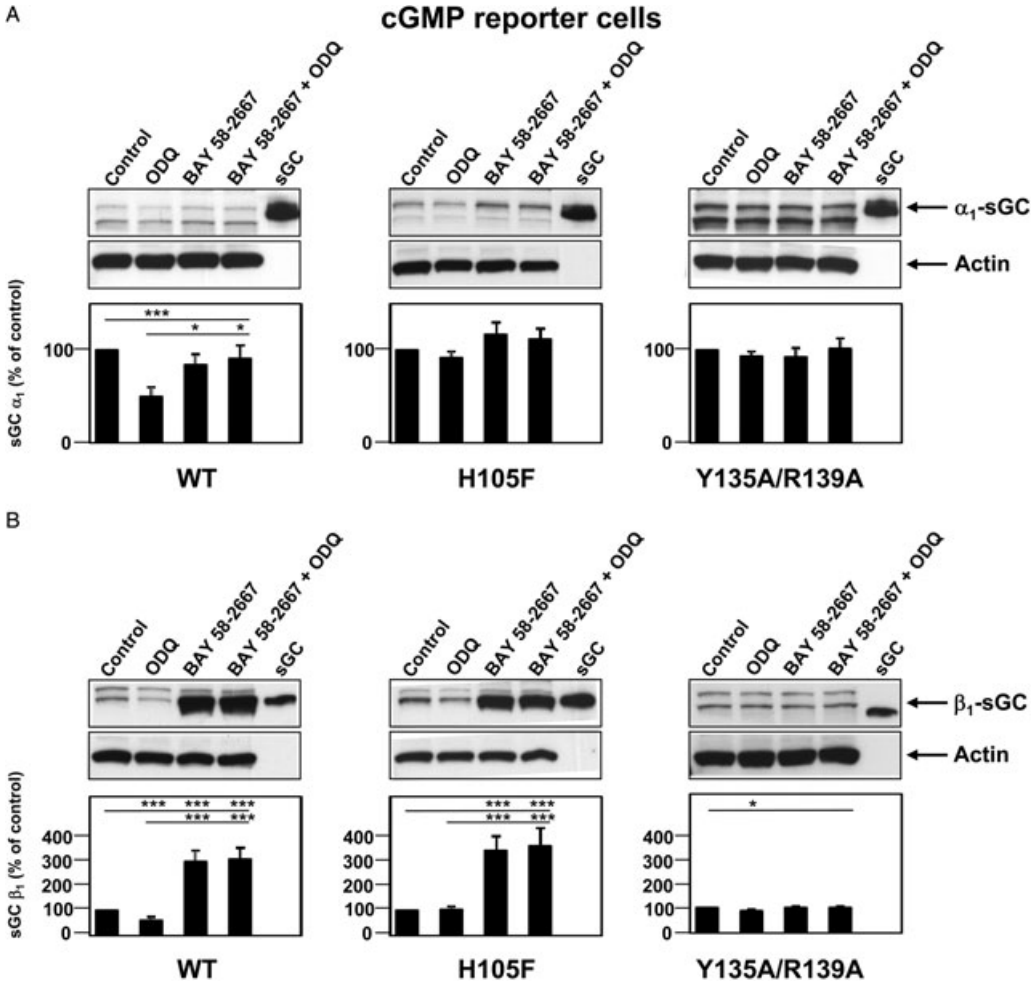

Figure 8 Effects of BAY 58-2667 on protein levels under normal and oxidative conditions of WT, α_1/β_1H105F sGC and α_1/β_1Y135A/R139A sGC. cGMP reporter cells were incubated with 10 μmol·L^{-1} ODQ or 10 μmol·L^{-1} BAY 58-2667 alone or combined for 24 h as indicated. (A) Representative Western blots of α_1 sGC and actin as a loading control (upper panel). Purified rat sGC was used as control. α_1 sGC protein levels as determined by densitometric measurement are shown in the lower panel. (B) Representative Western blots of β_1 sGC and actin as a loading control (upper panel). Purified rat sGC was used as control. β_1 sGC protein levels as determined by densitometric measurement are shown in the lower panel. sGC protein levels are normalized to the respective control, which was set as 100% (means ± SEM of three to eight independent experiments). *P < 0.05, **P < 0.01, ***P < 0.005: Student's *t*-test. BAY 58-2667, 4-[((4-carboxybutyl){2-[(4-phenethylbenzyl)oxy]phenethyl}amino)methyl[benzoic]acid; ODQ, 1H-(1,2,4)-oxadiazolo[4,3-*a*]quinoxalin-1-one; SEM, standard error or the mean; sGC, soluble guanylyl cyclase; WT, wildtype.

from the enzyme. The results obtained with HMR 1766 were less clear; the relative low affinity of this compound prevented full displacement of radioactively labelled BAY 58-2667 from the enzyme. Nevertheless, at high micromolar concentrations, HMR 1766 reduced ^{3}H-BAY 58-2667 binding to 67%, suggesting that HMR 1766, BAY 58-2667 and Zn-PPIX bind to the same or at least partially overlapping binding sites. The concentration of HMR 1766 used for this study is higher than the concentration used by Schindler *et al*. but was chosen to achieve similar sGC activation by HMR 1766 and BAY 58-2667. This difference in sGC sensitivity might be due to the facts that we used recombinant rat sGC expressed in and purified from a baculovirus/Sf9 insect cell system, instead of native bovine sGC. Heterologous expression in insect cells might result in an sGC that lacks certain post-translational modifications such as phosphorylation, which might affect sGC activity (Meurer *et al*., 2005).

Using Western blot analysis, the effect of these compounds on sGC protein levels upon haem oxidation by the sGC inhibitor, ODQ, was established. Long-term treatment of ECs and cGMP reporter cells with ODQ resulted in dramatically reduced protein levels of both sGC subunits, as observed for other primary cells (Stasch *et al*., 2006). This oxidation-induced degradation becomes prominent when the incubation time exceeds 2 h, whereas short-term incubations had no effect on sGC protein levels (Stasch *et al*., 2006). BAY 58-2667 and Zn-PPIX prevented this oxidation-induced decrease. In agreement with previous findings (Stasch *et al*., 2006), protein levels remained unchanged in cells treated with the sGC stimulator BAY 41-2272, suggesting that signalling events downstream of cGMP are not likely to be involved in sGC stabilization. Our results obtained with HMR 1766 were very surprising. Despite the fact that both BAY 58-2667 and HMR 1766 appear to

interact with the sGC haem pocket, HMR 1766 did not show any protective effect on sGC protein levels.

The differences in protein levels under normal and haem-oxidizing conditions reflected the observed changes in sGC activity. Short-term incubation with 10 $\mu mol \cdot L^{-1}$ ODQ has been shown to potentiate BAY 58-2667-induced sGC activation at most, indicating that the majority of cellular sGC is converted into the oxidized/haem-free state, which can be activated by BAY 58-2667. As no impact on sGC protein levels has been reported for short-term ODQ incubations, the maximal BAY 58-2667-induced sGC activation upon short-term ODQ treatment compared with the combined long- and short-term ODQ incubation should reflect the decrease in sGC protein levels. Under this condition, a reduction of maximal sGC activation by BAY 58-2667 of 69% (129 to 82-fold activation) was observed, matching the observed reduction in sGC protein levels of 60% for α_1 sGC and 36% of β_1 sGC.

As shown in Figure 3, both BAY 41-2272 and HMR 1766 were unable to prevent oxidation-induced degradation of sGC. Therefore, we expected similar results for BAY 41-2272- or HMR 1766-induced sGC activity.

BAY 41-2272 activated sGC 115-fold under control conditions (Figure 5B). Combination of short- and long-term treatment reduced sGC activation to 7%. This is even lower than protein levels in ODQ and BAY 41-2272-treated cells, which were decreased by 57% (α_1 sGC) and 43% (β_1 sGC).

But, in contrast to BAY 41-2272, HMR 1766-induced sGC activity was unchanged or only slightly diminished compared with normal conditions. This discrepancy might be due to technical limitations, as the strong activation of sGC at high concentrations of HMR 1766 resulted in RLUs at the limit of detection. Although the addition of ODQ slightly increases the amount of RLUs, the expected HMR 1766 plus ODQ-induced maximum activation can presumably not be measured. Again, the highest concentration of HMR 1766 chosen for sGC activity measurements was higher than the concentrations used by Schindler *et al.* (2006). The differences in efficacy might be explained by the use of different cell lines or primary cells.

Comparison of Figures 5A and 6A shows that ECs were not stimulated to the same extend as cGMP reporter cells under control conditions. This might be due to the different cell types. In ECs, compared with cGMP reporter cells, the low BAY 58-2667-induced stimulation might reflect a small pool of naturally oxidized/haem-free sGC. On the other hand, this would argue for a bigger pool of haem-free/oxidized sGC in cGMP reporter cells compared with ECs. Mingone *et al.* (2006) showed that the levels of haem precursors (e.g. 5-amino-laevulinic acid) directly impact on haem synthesis and, as a result, in the relative amount of NO-sensitive, haem-containing sGC. As the cGMP reporter cells express sGC at very high levels, it might be possible that the native cellular haem synthesis is not able to match the needs of this artificially high expression, resulting in increased relative amounts of BAY 58-2667-sensitive, haem-free enzyme. Further studies applying haemin or 5-amino-laevulinic-acid might be able to shed light on this question. Wolin (2009) has shown that the haem precursor PPIX accumulates in vascular tissue incubated with 5-amino-laevulinic acid and thereby stimulates sGC and induces pulmonary artery relaxation.

In ECs, a dramatic increase in sGC activator-induced activation of sGC following long-term treatment with ODQ was observed. The haem-free state of sGC is preferentially targeted by sGC activators (Stasch *et al.*, 2006; Roy *et al.*, 2008). Moreover, haem loss in only a small proportion of sGC pool results in a dramatic increase of BAY 58-2667-induced sGC activity (Roy *et al.*, 2008). In contrast, only a small decrease of BAY 41-2272-induced activation of ODQ pretreated sGC was observed, which might suggest a receptor reserve.

Protein levels of BAY 41-2272 and ODQ-treated ECs and cGMP reporter cells decreased by about 50% and reflected the measured sGC activity. In ECs, BAY 41-2272-induced activity was lowered by about 25%, and the corresponding EC_{50} was increased to about 25%. In cGMP reporter cells, sGC activity was diminished to an even greater extent.

In summary, BAY 58-2667 and Zn-PPIX but not BAY 41-2272 prevented sGC from oxidation-induced degradation. The measured sGC activity reflected the observed changes in protein levels. Importantly, the different sGC activators showed an unexpectedly different profile with respect to sGC protein and activity levels. Both haem-independent sGC activators, BAY 58-2667 and HMR 1766, induced sGC activation under normal and haem-oxidizing conditions, but only BAY 58-2667 was able to prevent oxidation-induced enzyme degradation. This molecular characteristic might be explained by different levels of haem mimicry and overlapping but not identical binding sites.

The sGC stabilizing ligands, BAY 58-2667 and Zn-PPIX, have the same chemical motif as haem and bind to the haem site with much higher affinity than the native prosthetic group. Furthermore, mutation analyses and structural models showed that both ligands interact with the haem-binding residues Y135 and R139 of the β_1 subunit (Schmidt *et al.*, 2004).

To investigate the interaction of the two haem-independent sGC activators, BAY 58-2667 and HMR 1766, the activation patterns of these compounds have been recorded with different sGC mutations that are known to effect haem-binding. Mutation of the axial haem ligand β_1H105F has been shown to cause the expression of haem-free enzyme (Foerster *et al.*, 1996), although this mutation did not preclude subsequent reconstitution of the enzyme with porphyrins (Schmidt *et al.*, 2004). In contrast, the soluble mutant β_1Y135A/R139A, which lacks the essential haem-binding residues, cannot be reconstituted with PPIX (Schmidt *et al.*, 2004). Furthermore, Y135 and R139 were also identified as binding sites for BAY 58-2667 as the double mutant is no longer activated by BAY 58-2667 (Schmidt *et al.*, 2004).

This double mutant α_1/β_1Y135A/R139A was used to determine whether both BAY 58-2667 and HMR 1766 activate sGC by binding to these residues within the haem binding motif (Schmidt *et al.*, 2004).

When measuring sGC activity in cGMP reporter cells expressing these haem-free sGC variants, HMR 1766 and BAY 58-2667 apparently did not bind to the same residues. Although BAY 58-2667 is able to strongly activate the haem-free sGC mutant H105F, it was not able to induce any activation of the double mutant α_1/β_1Y135A/R139A, indicating that these residues are crucially important for BAY 58-2667 binding, as shown earlier (Schmidt *et al.*, 2004). In contrast,

HMR 1766 still activates the double mutant although its activation is diminished compared with WT sGC. These data clearly suggest that Y135 and R139 do not affect binding of HMR 1766 in the same way or extent as they affect binding of BAY 58-2667 or haem. Zhou *et al.* (2008) used docking simulations based on the sGC structure of *Nostoc sp* to identify putative regions through which HMR 1766 interacts with sGC. Contrary to our findings in living cells, they postulated Y135 and R139 as binding partners of HMR 1766, suggesting a BAY 58-2667-like binding mode.

Considering the results from the receptor binding assay, which showed that HMR 1766 competes with BAY 58-2667 only at high concentrations whereas it can be readily replaced by low amounts of Zn-PPIX, it becomes evident that BAY 58-2667 bears noticeably more resemblance to haem. In contrast, HMR 1766 seems to interact with different residues from those interacting with BAY 58-2667, although their binding sites might overlap at least partially.

Basing on these findings, we hypothesized that the different binding modes to the haem pocket might be responsible for the differences between HMR 1766 and BAY 58-2667 with respect to protection of sGC from oxidation-induced degradation. When using the same sGC mutants described above in Western blots, it became apparent that the protective function of BAY 58-2667 is also mediated by the haem-like occupation of the haem. This protection can only be observed for WT and β_1H105F sGC, which are activated by BAY 58-2667, but not for β_1Y135A/R139A sGC, which neither is activated by BAY 58-2667 nor can be reconstituted with PPIX (Schmidt *et al.*, 2004). HMR 1766 was not used in this set of experiments because of its lack of protective or stabilizing properties, which was already demonstrated in WT-sGC-expressing cells, and HMR 1766-induced activity in Y135A/R139A sGC-expressing cells showed its different binding mode.

Another difference becomes obvious by comparing α_1 and β_1 sGC levels: Oxidation-induced degradation of the α_1 subunit is not prevented by BAY 58-2667 and Zn-PPIX to the same extent as observed for the β_1 subunit. Based on our data with β_1H105F sGC, it is more likely that the β subunit is predominantly affected by ODQ. Incubation with ODQ does not lead to a significant decrease in sGC protein levels in cells expressing β_1H105F, as observed in cells expressing WT sGC. In addition, the BAY 58-2667 activation profile of β_1H105F expressing cells resembles the pattern of BAY 58-2667-induced sGC activity in ODQ treated WT cells. In agreement with Stasch *et al.* (2006), we assume that the physiological turnover of α_1 sGC is not affected due to the lack of haem binding, and, thus, any changes that are mediated via the haem binding site do not apply to α_1 sGC directly. The changes we observed for α_1 sGC protein levels may rather be based on counter regulatory mechanisms as described for the α_1 and α_2 knockout mice (Mergia *et al.*, 2006). Here, deletion of α subunits results in a concomitant decrease of β_1 protein levels. Moreover, Friebe *et al.* (2007) demonstrated that β_1 knockout mice lack the α_1 subunit. We suppose that the observed changes underlie a similar mechanism of counter regulation, which results in decreased α_1 protein levels when β_1 subunits are depleted due to oxidation. However, instability of single α_1 subunits cannot be an explanation for the

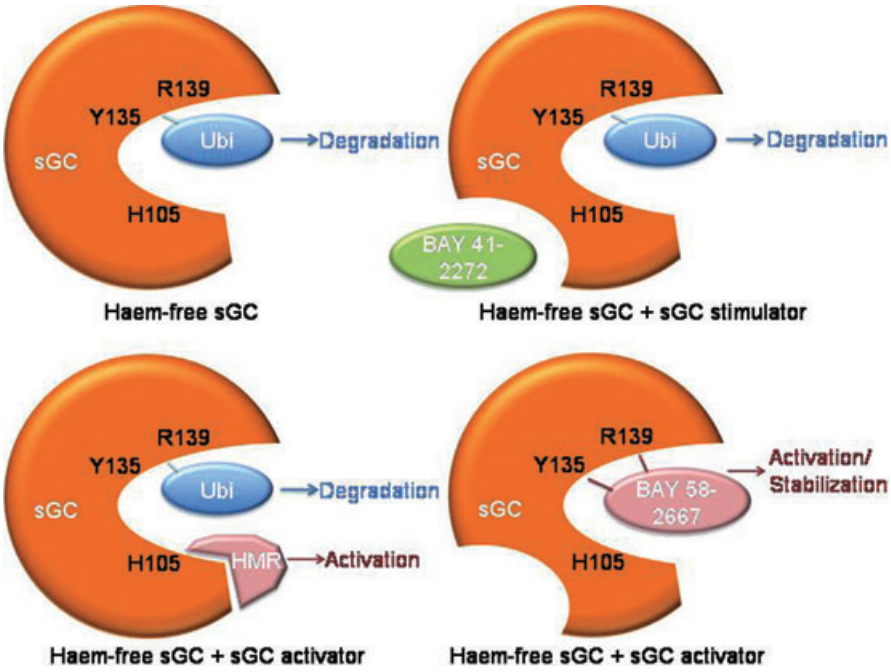

Figure 9 Model of the role of sGC's haem group and sGC targeting compounds in protection of sGC. Under haem-oxidizing conditions such as oxidative stress, binding of the sGC activator BAY 58-2667 stabilizes sGC and thereby protects sGC from degradation. In contrast, binding of the sGC activator HMR 1766 or the sGC stimulator BAY 41-2272 cannot stabilize sGC and therefore sGC like haem-free sGC is ubiquitinated and degraded. BAY 41-2272, 5-cyclopropyl-2-[1-(2-fluoro-benzyl)-1*H*-pyrazolo[3,4-*b*]pyridin-3-yl]-pyrimidin-4-ylamine; BAY 58-2667, 4-[((4-carboxybutyl){2-[(4-phenethylbenzyl)oxy]phenethyl}amino)methyl[benzoic]acid; sGC, soluble guanylyl cyclase; HMR 1766, 5-chloro-2-(5-chloro-thiophene-2-sulphonylamino-*N*-(4-(morpholine-4-sulphonyl)-phenyl)-benzamide sodium salt.

decrease in α_1 sGC protein levels, as homodimers can form and are stable (Zabel *et al*., 1999).

In summary, our results show that BAY 58-2667 and Zn-PPIX but neither BAY 41-2272 nor HMR 1766 prevent oxidation-induced degradation of sGC and decreased sGC activity. BAY 58-2667's protective effect depends on high-affinity binding to the haem-binding pocket in a manner that reassembles the native prosthetic group including the interaction with the haem binding motif Y-x-S-x-R. HMR 1766 lacks these properties, making it a distinct class of sGC activators. The results of this study are summarized in Figure 9. To our knowledge, BAY 58-2667 is thus the first pharmacological enzyme ligand, which, in addition to activating, also stabilizes its own target. As cardiovascular diseases are associated with increased levels of oxidative stress, it can be expected that the relative amount of haem-oxidized/haem-free sGC is increased under pathophysiological conditions. This view is in agreement to results obtained in a clinical trial with patients suffering from acute decompensated heart failure, which suggest the presence of a pool of haem-free/oxidized sGC in humans (Schmidt *et al*., 2009). This imbalance would be translated into decreased sGC protein levels due to accelerated degradation of the oxidation-impaired enzyme. The sGC stabilizing features of BAY 58-2667 described here might help to overcome this imbalance by preventing sGC from degradation and thus improving cardiovascular disease. Further pharmacological and clinical studies with sGC activators will provide more information on the *in vivo* efficacy and effects in the treatment of cardiovascular diseases.

Conflict of interest

LS Hoffmann, S Schaefer, Y Keim and JP Stasch are fulltime employees of Bayer HealthCare.

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