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# Mechanisms of nitric oxide independent activation of soluble guanylyl cyclase

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**Abstract**

The heterodimeric heme-protein soluble guanylyl cyclase (sGC) is the only proven receptor for nitric oxide (NO). Recently, two different types of NO-independent soluble guanylyl cyclase stimulators have been discovered. The heme-dependent stimulator 2-[1-[2-fluorophenyl)methyl]-1*H*-pyrazolo[3,4-b]pyridin-3-yl]-5(4-morpholinyl)-4,6-pyrimidinediamine (BAY 41-8543) stimulates the enzyme in a synergistic fashion when combined with NO, requires the presence of the heme group and can be blocked by the soluble guanylyl cyclase inhibitor 1*H*-(1,2,4)-Oxadiazole-(4,3-a)-quinoxalin-1-one (ODQ). The heme-independent activator 4-[((4-carboxybutyl){2-[(4-phenethyl-benzol) oxy]phenethyl}amino)methyl[benzoic]acid (BAY 58-2667) activates soluble guanylyl cyclase even in the presence of ODQ or rendered heme-deficient. In the present study, BAY 41-8543, BAY 58-2667 and NO strongly increased $V_{max}$. Combination of BAY 58-2667 and NO increased $V_{max}$ in an additive manner, whereas the synergistic effect of BAY 41-8543 and NO on enzyme activation was reflected in an overadditive increase of $V_{max}$. ODQ potentiated $V_{max}$ of BAY 58-2667-stimulated soluble guanylyl cyclase. BAY 41-8543 prolonged the half-life of the nitrosyl–heme complex of NO-activated enzyme, an effect that was not observed with BAY 58-2667. These results show the different activation patterns of both compounds and demonstrate their value as tools to investigate the mechanisms that underlie soluble guanylyl cyclase activation.
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*Keywords:* BAY 41-8543; BAY 58-2667; Kinetics; Heme; Nitric oxide (NO); Soluble guanylyl cyclase

## 1. Introduction

Guanylyl cyclases [GTP pyrophosphat lyase (cyclizing) E.C. 4.6.1.2.] are responsible for the synthesis of the second messenger cGMP which mediates many physiological functions such as vasorelaxation, inhibition of platelet aggregation, neurotransmission and light transduction in the retina (Ignarro et al., 1987; Garthwaite et al., 1988; Moncada and Higgs, 1995). Soluble guanylyl cyclase (sGC) is the only proven receptor for the gaseous ligand nitric oxide (NO). The heterodimeric enzyme consists of an α- and β-subunit. Two α-subunits, $\alpha_1$ and $\alpha_2$, and two β-subunits, $\beta_1$ and $\beta_2$, were characterized previously (for review: Hobbs, 2000). Both α-subunits can form functional identical heterodimers with the $\beta_1$-subunit and were detected at the protein level in various tissues (Russwurm et al., 1998). As prosthetic group, soluble guanylyl cyclase contains one heme per dimer that is crucial for the binding of NO and therefore for the activation of the enzyme (Wedel et al., 1994; Stone and Marletta, 1996). Although this pathway of soluble guanylyl cyclase activation is generally accepted, there are still controversial discussions about the exact mechanism (Zhao et al., 1999; Bellamy et al., 2002; Ballou et al., 2002). Beyond this major exogenous and endogenous mechanism of soluble guanylyl cyclase activation, there is some evidence for modulation of the activity of soluble guanylyl cyclase by translocation, cations and adenine nucleotides (Zabel et al., 2002; Parkinson et al., 1999; Ruiz-Stewart et al., 2002).

In recent years, a non-gaseous soluble guanylyl cyclase activating ligand, 3-(5′ -hydroxymethyl-2′ furyl)-1-benzyl-indazol (YC-1), has been described (Wu et al., 1995; Friebe et al., 1996; Mülsch et al., 1997). YC-1 shows an unique characteristic in soluble guanylyl cyclase activation: the heme moiety is essential for the activation of soluble guanylyl cyclase by YC-1 and removal of this prosthetic

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group or oxidation to its ferric form by the soluble guanylyl cyclase inhibitor 1*H*-(1,2,4)-oxadiazole-(4,3-a)-quinoxalin-1-one (ODQ) leads to an enzyme that is no longer responsive to NO or YC-1 (Schrammel et al., 1996; Hoenicka et al., 1999; Zhao et al., 2000). Furthermore, and in contrast to the only weak stimulation of the purified enzyme by YC-1, this compound shows a strong synergism with NO, making it a valuable tool for exploring the mechanism of soluble guanylyl cyclase activation. Very recently, we discovered 5-cyclopropyl-2-[1-(2-fluoro-benzyl)-1*H*-pyrazolo[3,4-b]pyridin-3-yl]-pyrimidin-4-ylamine (BAY 41-2272) and 2-[1-[2-fluorophenyl)methyl]-1*H*-pyrazolo[3,4-b]pyridin-3-yl]-5(4-morpholinyl)-4,6-pyrimidinediamine (BAY 41-8543). These soluble guanylyl cyclase stimulating compounds display similar characteristics as YC-1 but with a higher potency of about two to three orders of magnitude and no phosphodiesterase inhibitory activity (Stasch et al., 2001, 2002a,b; Hobbs, 2002). Moreover, we discovered heme-independent soluble guanylyl cyclase activators, such as 4-[((4-carboxybutyl){2-[(4-phenethylbenzol) oxy]phenethyl}amino)methyl[benzoic]acid (BAY 58-2667), that behave in a manner completely different from all known soluble guanylyl cyclase stimulators (Stasch et al., 2002c; Hobbs, 2002). BAY 58-2667 shows an additive effect on soluble guanylyl cyclase activation in combination with NO and activates the enzyme even after it has been oxidized by the soluble guanylyl cyclase inhibitor ODQ or rendered heme deficient. The present study was performed to compare the activation profile, the kinetic parameters and the half-life of the nitrosyl–heme complex at the soluble guanylyl cyclase in the presence of the heme-dependent stimulator BAY 41-8543 and the heme-independent activator BAY 58-2667. Our findings demonstrate that BAY 41-8543 and BAY 58-2667 act via different mechanisms of soluble guanylyl cyclase activation and may therefore serve as valuable tools to elucidate structures and functions of novel regulatory sites at the soluble guanylyl cyclase.

## 2. Materials and methods

### 2.1. Substances

BAY 41-8543 and BAY 58-2667 were synthesized as described previously (Straub et al., 1998; Alonso-Alija et al., 2001). 2-(*N*,*N*-Diethylamino)-diazenolate-2-oxide (DEA/NO) and 2-phenyl-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide (PTIO) were purchased from Alexis Biochemicals (San Diego, USA). All other chemicals of analytical grade were obtained from Sigma (Taufkirchen, Germany).

### 2.2. Spectroscopic studies

UV/VIS spectra were recorded from 300 to 600 nm on a Beckman DU 640 spectrophotometer as reported earlier (Hoenicka et al., 1999). NO was introduced via an aqueous solution of DEA/NO. A 100 mM stock solution of BAY 41-8543 and BAY 58-2667 was prepared and added in a final concentration of 10 μM, resulting in a final dimethylsulfoxide (DMSO) concentration of 0.1% (v/v).

### 2.3. Purification and soluble guanylyl cyclase activity assay

We expressed and purified soluble guanylyl cyclase using a baculovirus/SF9 expression system according to published protocols (Hoenicka et al., 1999). Enzyme activity was measured by formation of [$^{32}$P]-cGMP from [α-$^{32}$P]-GTP in the presence of $Mg^{2+}$ as described (Hoenicka et al., 1999). Briefly, soluble guanylyl cyclase was incubated with various compounds for 10 min at 37 °C in incubation buffer (50 mM triethanolamine (TEA), 100 μM EGTA, 1 mM 3-isobutyl-1-methylxanthine (IBMX), 1 mM dithiothreitol (DTT), 1 mM cGMP, 200 μM GTP, 5 mM creatine phosphate, 12.5 U/ml creatine phosphokinase, 3 mM $MgCl_2$, 1 mg/ml bovine serum albumin, pH 7.5). The reaction was stopped by coprecipitation of 5′ -nucleotides with $ZnCO_3$. Following centrifugation (5 min, 4 °C, 2800 × *g*) [$^{32}$P]-cGMP was isolated from the supernatant by chromatography on neutral alumina columns by elution with 10 ml 100 mM Tris (pH 7.5). The amount of [$^{32}$P]-cGMP formed was determined in a PW 4700 liquid scintillation counter (Raytest, Straubenhardt, Germany). For the measurement of the kinetic parameters $K_m$ and $V_{max}$, the soluble guanylyl cyclase assay was performed with increasing concentrations of GTP ranging from 12.5 μM over 25, 50, 100, 200 to 400 μM. All measurements were performed in duplicate and were repeated three times unless otherwise indicated. The highest DMSO concentration in the test was 1% (v/v) and did not elicit any effect per se on cGMP production.

### 2.4. Sensitization of soluble guanylyl cyclase towards NO

The determination of the half-life of the nitrosyl–heme complex was performed as described (Russwurm et al., 2002). Briefly, purified soluble guanylyl cyclase (2 μg) was incubated in incubation buffer with a total volume of 20 μl containing 50 μM DEA/NO for 165 s at 37 °C. After addition of GTP and $MgCl_2$ (final concentration 40 nM GTP, 400 nM $MgCl_2$) and incubation for further 15 s, the sample was diluted 100-fold in incubation buffer (37 °C) containing [α-$^{32}$P]-GTP and 50 μM PTIO as NO scavenger. Aliquots (100 μl) were withdrawn at the time points indicated. The reaction was stopped by addition of 100 mM $ZnCO_3$. The amount of formed [$^{32}$P]-cGMP was measured as outlined above. To measure the effect of the test compounds on the stabilization of the nitrosyl–heme complex, soluble guanylyl cyclase was preincubated with 50 μM DEA/NO as described above, alone or in the presence of 10 μM BAY 41-8543 or 1 μM BAY 58-2667 before dilution. For the determination of the half-life of the nitrosyl–heme complex in the presence of BAY 41-8543, the assay was performed with 0.1 μg soluble

guanylyl cyclase (due to the strong synergistic activation of soluble guanylyl cyclase in the presence of BAY 41-8543). Nonlinear regression was calculated as reported by Russwurm et al. (2002) using Graphpad Prism (GraphPad Software, San Diego, USA).

## 3. Results

### 3.1. Spectroscopic studies

To determine if BAY 41-8543 or BAY 58-2667 (Fig. 1A and B) shows any direct interaction with the prosthetic heme moiety, we recorded UV/VIS spectra of soluble guanylyl cyclase under various conditions. As shown in Fig. 2A and B, the native soluble guanylyl cyclase showed the characteristic Soret peak at 431 nm shifted to 398 nm after incubation with DEA/NO, indicating the formation of the five-coordinated nitrosyl–heme complex (Hoenicka et al., 1999). Incubation with BAY 41-8543 (Fig. 2A) or BAY 58-2667 (Fig. 2B) did not induce a shift of the Soret peak of the native or NO-activated form of soluble guanylyl cyclase.

### 3.2. Activation of soluble guanylyl cyclase

We determined the activation profile of BAY 41-8543 and BAY 58-2667 at the native enzyme as well as in the presence of NO and ODQ. The basal activity of the enzyme was in this study $158 \pm -13$ and $111 \pm 11$ nmol $min^{-1}$ $mg^{-1}$, respectively. Incubation of the native soluble guanylyl cyclase with BAY 41-8543 led to a concentration-dependent activation of the enzyme from 10 nM to 100 μM up to 66-fold. In the presence of 10 nM of the NO donor 3-morpholinosydnonimine (SIN-1), a concentration which shows only negligible effect on soluble guanylyl cyclase activation (2.5-fold), a strong synergism could be observed in combination with BAY 41-8543 over a wide range of concentration with a maximal effect of 202-fold at higher concentration of SIN-1 (100 nM; Fig. 3A). Addition of the soluble guanylyl cyclase inhibitor ODQ (10 μM) to the enzyme resulted in a 71% inhibition of the BAY 41-8543-activated soluble guanylyl cyclase from 66-fold to 19-fold vs. basal (Fig. 3A).

Incubation of soluble guanylyl cyclase with BAY 58-2667 led to a concentration-dependent activation of the enzyme from 1 nM to 10 μM up to 30-fold. In combination

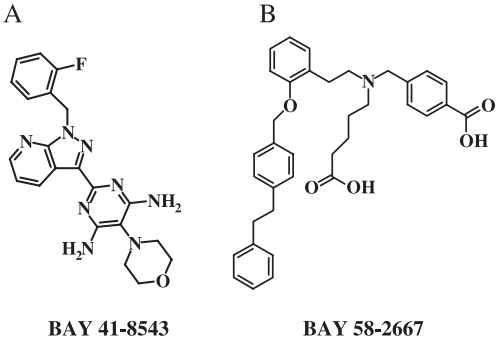


Fig. 1. Heme-dependent soluble guanylyl cyclase stimulator BAY 41-8543 (A) and heme-independent soluble guanylyl cyclase activator BAY 58-2667 (B).

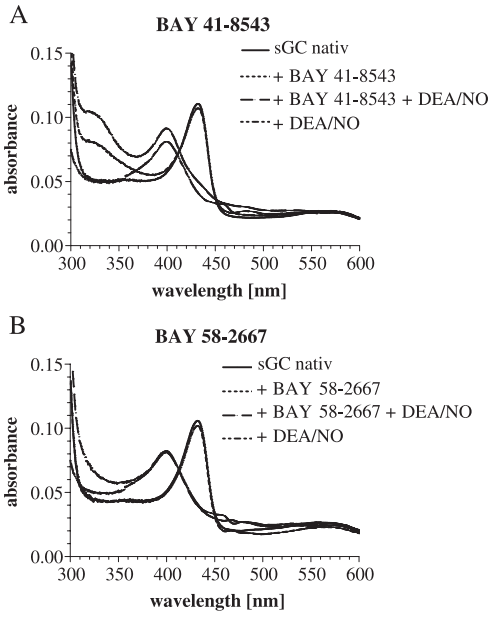


Fig. 2. UV/VIS spectra of the native and DEA/NO stimulated soluble guanylyl cyclase (15.5 μg) in the absence and presence of 100 μM BAY 41-8543 (A) and 100 μM BAY 58-2667 (B).

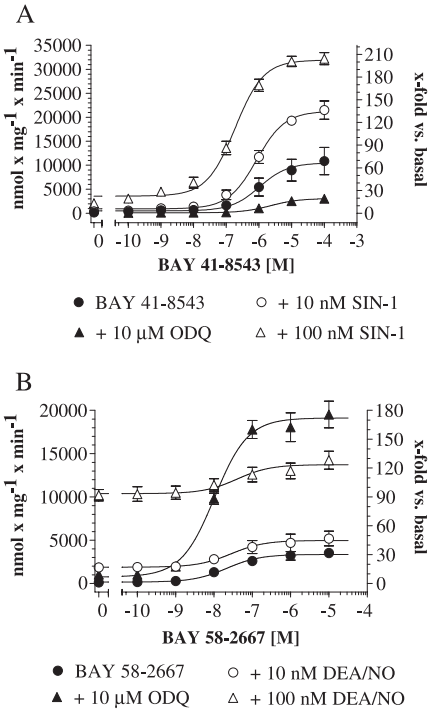


Fig. 3. Specific activity of soluble guanylyl cyclase after incubation with BAY 41-8543 (A) and BAY 58-2667 (B) combined with two concentrations of NO donors (10, 100 nM) or ODQ (10 μM). Data are mean ± S.E.M. from four independent experiments that were performed in duplicate.

with the NO donor DEA/NO, an only additive effect of BAY 58-2667 was observed at the various test concentrations (Fig. 3B). The NO donor DEA/NO increased the basal enzyme activity up to 17-fold (10 nM) and 94-fold (100 nM), respectively. In the presence of the highest concentration of BAY 58-2667 (10 μM), enzyme activity was elevated after addition of DEA/NO (10, 100 nM) from basal 30-fold to 45-fold and to 124-fold underlining the additive effect. Incubation with the soluble guanylyl cyclase inhibitor ODQ (10 μM) led to a strong potentiation of the soluble guanylyl cyclase activation by BAY 58-2667 from 30-fold to 173-fold (Fig. 3B). Soluble guanylyl cyclase was incubated with increasing concentrations of BAY 41-8543 in the absence or presence of 1 μM BAY 58-2667. Incubation with BAY 41-8543 resulted in a dose-dependent increase of the specific enzyme activity from $1612 \pm 349$ (0.1 μM), $4254 \pm 231$ (1 μM), $10708 \pm 1138$ (10 μM) to $13668 \pm 1294$ (100 μM) nmol $min^{-1}$ $mg^{-1}$ in the absence, and $6561 \pm 1033$ (0.1 μM), $8185 \pm 1249$ (1 μM), $13653 \pm 1923$ (10 μM) and $17582 \pm 1270$ (100 μM) nmol $min^{-1}$ $mg^{-1}$ in the presence of 1 μM BAY 58-2667.

### 3.3. Determination of kinetic parameters

For the investigation of the nature of soluble guanylyl cyclase activation, we determined the kinetic parameters $K_m$ and $V_{max}$ at the basal state as well as in the presence of NO, BAY 41-8543 and BAY 58-2667. At the basal state we calculated a $K_m$ of 74 μM and a $V_{max}$ of 0.134 μmol $min^{-1}$ $mg^{-1}$ (Figs. 4A and 5A, Table 1). Addition of BAY 41-8543 (10 μM) or BAY 58-2667 (100 nM) led to an unaffected or slightly decreased $K_m$ value of 79 and 56 μM, respectively, whereas the addition of low concentrations of DEA/NO (10 nM) led to a slight increase of $K_m$ values of the basal and both activated states (Table 1). Addition of ODQ (10 μM) resulted in a small increase of $K_m$ in BAY 41-8543- and BAY 58-2667-activated states as shown in Figs. 4 and 5.

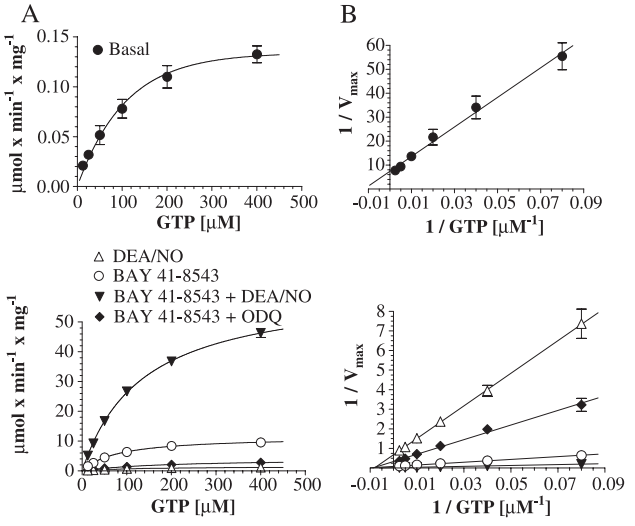


Fig. 4. Determination of kinetic parameters $K_m$ and $V_{max}$ under different stimulated conditions. Nonlinear regression of the specific soluble guanylyl cyclase activity (μmol cGMP $min^{-1}$ $mg^{-1}$) vs. GTP concentration (μM) is shown in A for basal state, DEA/NO (10 nM) and BAY 41-8543 (10 μM). The same data as Lineweaver–Burk plot is shown in B. Data are mean ± S.E.M. from three to six independent experiments that were performed in duplicate.

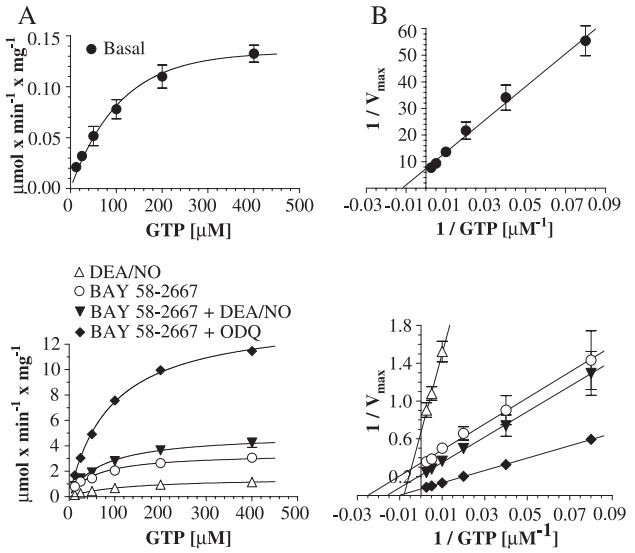


Fig. 5. Determination of kinetic parameters $K_m$ and $V_{max}$ under different stimulated conditions. Nonlinear regression of the specific soluble guanylyl cyclase activity (μmol cGMP $min^{-1}$ $mg^{-1}$) vs. GTP concentration (μM) is shown for basal state, DEA/NO (10 nM) and BAY 58-2667 (100 nM) in A. The same data as Lineweaver–Burk plot is shown in B. Data are mean ± S.E.M. from three to six independent experiments that were performed in duplicate.

Table 1

| | $K_m^a$ | $V_{max}^b$ | $K_m^c$ | $V_{max}^d$ |
|---|---|---|---|---|
| Basal | 74.2 ± 9.2 | 0.134 ± 0.02 | 1 | 1 |
| DEA/NO | 119.4 ± 16.7 | 1.49 ± 0.08 | 1.6 | 11.1 |
| BAY 41-8543 | 79.3 ± 14.8 | 11.53 ± 0.77 | 1.1 | 86.0 |
| BAY 41-8543 + DEA/NO | 135.5 ± 7.3 | 62.0 ± 1.40 | 1.8 | 462.7 |
| BAY 41-8543 + ODQ | 183.1 ± 11.3 | 4.08 ± 0.12 | 2.5 | 30.4 |
| BAY 58-2667 | 56.1 ± 13.1 | 3.40 ± 0.26 | 0.8 | 25.4 |
| BAY 58-2667 + DEA/NO | 70.9 ± 13.6 | 4.93 ± 0.33 | 1.0 | 36.8 |
| BAY 58-2667 + ODQ | 90.6 ± 4.0 | 14.23 ± 0.23 | 1.2 | 106.2 |

Determination of kinetic parameters $K_m$ and $V_{max}$ under different conditions of soluble guanylyl cyclase stimulation. Parameters determined from nonlinear regression of the specific enzyme activity vs. GTP concentration for basal, DEA/NO (10 nM), BAY 41-8543 (10 μM), BAY 58-2667 (100 nM) and ODQ (10 μM) stimulated conditions. Data are mean ± S.E.M. from four to eight independent experiments that were performed in duplicate.

[a] $K_m$ is the GTP-concentration in μM where the enzyme activity reaches 50% of its maximum.

[b] $V_{max}$ is the maximal specific katalytic rate in μmol cGMP $min^{-1}$ $mg^{-1}$.

[c] $K_m$ value expressed as factor compared to the mean basal $K_m$.

[d] $V_{max}$ value expressed as factor compared to the mean basal $V_{max}$.

The maximal catalytic rate $V_{max}$ changed dramatically after incubation with the various soluble guanylyl cyclase activating compounds. Incubation of soluble guanylyl cyclase with low concentration of DEA/NO (10 nM) led to a 11-fold increase of $V_{max}$, whereas a 86-fold increase of $V_{max}$ could be observed after addition of 10 μM BAY 41-8543. Concomitant with the findings of the activity assay, incubation of the enzyme with 10 μM BAY 41-8543 and 10 nM DEA/NO raised the $V_{max}$ value up to 462-fold compared to the basal state (Fig. 4, Table 1). Incubation with BAY 58-2667 (100 nM) led to an increase of $V_{max}$ of 25-fold in the absence and 36-fold in the presence of 10 nM DEA/NO, showing a strictly additive effect on the maximal catalytic rate (Fig. 5, Table 1). Reflecting the strong increase in cGMP formation observed in the soluble guanylyl cyclase activity assay (Fig. 3B, Table 1), addition of BAY 58-2667 in the presence of the soluble guanylyl cyclase inhibitor ODQ (10 μM) resulted in an increase of $V_{max}$ up to 106-fold compared to basal state.

### 3.4. Deactivation of soluble guanylyl cyclase

To further elucidate the different characteristics of soluble guanylyl cyclase activation through BAY 41-8543 or BAY 58-2667 in combination with NO, we determined the half-life of the nitrosyl–heme complex for the native NO-activated soluble guanylyl cyclase as well as in the presence of both test compounds. As shown in Fig. 6A, the calculated half-life of the nitrosyl–heme complex for the native soluble guanylyl cyclase is 1.6 s. Preincubation with BAY 41-8543 (10 μM) resulted in a 220-fold longer half-life, suggesting that this compound stabilizes the nitrosyl–heme complex (Fig. 6C). Preincubation with BAY 58-2667 (1 μM) resulted in no distinct alteration (1.9-fold) of the half-life of the nitrosyl–heme complex (Fig. 6B).

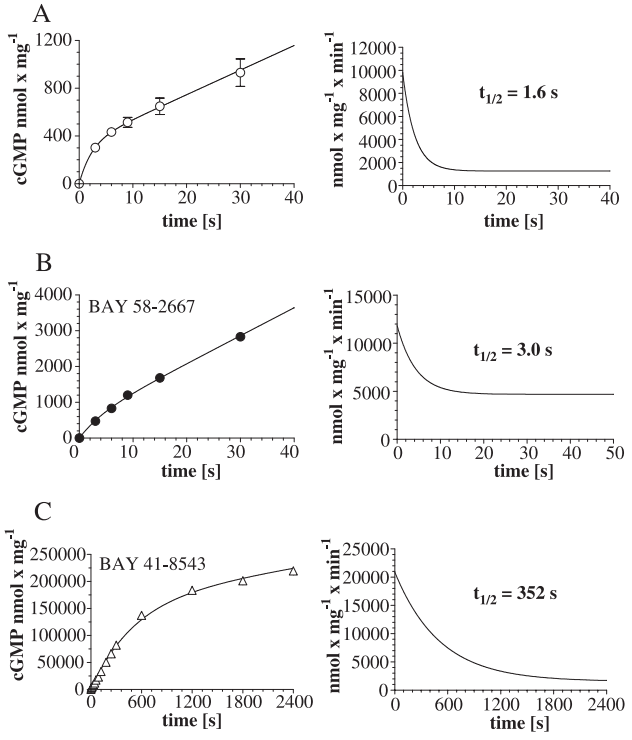


Fig. 6. Determination of the half-life of the nitrosyl-heme complex under basal conditions (A) and activated states. The alteration of the NO dissociation in the presence of BAY 58-2667 (1 μM) is shown in B whereas the results after incubation with BAY 41-8543 (10 μM) are shown in C. Data are mean ± S.E.M. from six independent experiments.

## 4. Discussion

Recently we identified and characterized two novel classes of non-NO-based activators of soluble guanylyl cyclase that offers new approaches for both the understanding of soluble guanylyl cyclase regulation and the treatment of cardiovascular diseases. The heme-dependent soluble guanylyl cyclase stimulators BAY 41-2272 and BAY 41-8543 show similar in vitro characteristics as the indazole derivative YC-1. However, they are about two to three orders of magnitude more potent in activating soluble guanylyl cyclase and, in contrast to YC-1, they display no phosphodiesterase inhibitory activity (Stasch et al., 2001, 2002a,b; Straub et al., 2001). There is evidence that these compounds interact with a recently located allosteric site at the soluble guanylyl cyclase (Stasch et al., 2001; Becker et al., 1999). The heme-independent soluble guanylyl cyclase activators, represented by BAY 58-2667, exhibit completely different characteristics in soluble guanylyl cyclase activation compared to the heme-dependent soluble guanylyl cyclase stimulators (Stasch et al., 2002c; Hobbs, 2002). BAY 58-2667 activates the native enzyme even in the presence of the soluble guanylyl cyclase inhibitor ODQ as well as the heme-deficient enzyme.

In this study, we characterized the two novel mechanisms of soluble guanylyl cyclase activation by measurement of the spectroscopic characteristics of the activated enzyme, the activation profile, the kinetic parameters and the prolongation of the half-life of the nitrosyl–heme complex at soluble guanylyl cyclase. First we determined a possible direct interaction of BAY 41-8543 or BAY 58-2667 with the central iron of the prosthetic heme moiety of soluble guanylyl cyclase by recording UV/VIS-spectra. BAY 41-8543 showed no alteration of the Soret peak in the basal or NO-activated state of soluble guanylyl cyclase as previously reported for both YC-1 and BAY 41-2272 (Friebe and Koesling, 1998; Stasch et al., 2001). BAY 58-2667 showed also no alteration of the Soret peak in the UV/VIS spectra in the basal and the NO-activated state of soluble guanylyl cyclase. These results suggest that both BAY 41-8543 and BAY 58-2667 do not interact directly with the iron core of the prosthetic heme moiety of soluble guanylyl cyclase. However, it cannot be excluded that both compounds interact with residues in close proximity to the heme binding side, resulting in small perturbations of this binding pocket. This question may be answered by further high resolution

spectroscopic analysis like Raman resonance spectra as previously performed with YC-1 (Denninger et al., 2000). In that earlier study, some minor alterations in the Fe-NO and also in the Fe-CO (carbon monoxide) stretching frequency were found in the presence of YC-1. According to the authors, this alteration was due to some minor conformational changes in the heme binding pocket after incubation with YC-1 (Denninger et al., 2000).

Heme-dependent stimulators such as YC-1, BAY 41-2272 or BAY 41-8543 are well characterized in the literature (Ko et al., 1994; Friebe and Koesling, 1998; Stasch et al., 2001, 2002a,b). Recently, a new class of heme-independent enzyme activators, represented by BAY 58-2667, has been identified (Stasch et al., 2002c). To characterize the properties of this new type of heme-independent activators, we compared the activation profiles of BAY 58-2667 and BAY 41-8543 at the soluble guanylyl cyclase under different NO concentrations as well as in the absence or presence of ODQ. Furthermore, we investigated the effect on the enzyme activation after incubation with both compounds. The heme-dependent soluble guanylyl cyclase stimulator BAY 41-8543 produced a dose-dependent enzyme activation and a strong synergism when combined with different NO concentrations. This is in agreement with earlier observations (Stasch et al., 2002a). Soluble guanylyl cyclase activation through BAY 41-8543 could be blocked by the soluble guanylyl cyclase inhibitor ODQ. Combination of BAY 41-8543 and BAY 58-2667 resulted in an additive effect on enzyme activation concomitant with the findings described recently that suggest two different and independent binding sites for both compounds at the enzyme (Stasch et al., 2002c; Schmidt et al., 2003).

BAY 58-2667 activated soluble guanylyl cyclase, over a wide range of concentrations and in contrast to BAY 41-8543, showed only an additive effect on the enzyme activation in the presence of different NO concentrations. Moreover, the soluble guanylyl cyclase inhibitor ODQ, which is known to oxidize the prosthetic heme moiety (Schrammel et al., 1996; Zhao et al., 2000), potentiated the enzyme activation induced by BAY 58-2667. As recently described, the oxidized and heme-deficient state of soluble guanylyl cyclase show the same strong activation in the presence of BAY 58-2667 (Stasch et al., 2002c). In addition, in a photoaffinity labeling study the same shift of the labeling pattern from the α- to the β-subunit of the enzyme was observed in both oxidized and heme-free soluble guanylyl cyclase (Stasch et al., 2002c). These findings suggest conformational changes in the structure of the enzyme by oxidation or removal of the prosthetic heme moiety. This structural changes may result in the recently described increased binding of BAY 58-2667 to soluble guanylyl cyclase and, as a consequence, to the increased responsiveness of the enzyme (Stasch et al., 2002c). The presence of the heme moiety in the native enzyme seems to interfere with these structural changes. To investigate if the central iron of the heme moiety is involved in this interference, soluble guanylyl cyclase was incubated with protoporphyrin IX combined with BAY 58-2667. The same interference as observed for the native heme moiety was found with protoporphyrin IX (data not shown). Therefore, the presence of heme or protoporphyrin IX seems to suppress the structural alterations of the enzyme that lead to the increased responsiveness of soluble guanylyl cyclase in the presence of BAY 58-2667.

Due to the differences in the activation profile, we determined the type of activation by measuring the kinetic parameters $K_m$ and $V_{max}$ for the basal state as well as in the presence of NO, BAY 41-8543 and BAY 58-2667. For the basal state of soluble guanylyl cyclase, we determined a $K_m$ of 74 μM and a $V_{max}$ of 0.13 μmol $min^{-1}$ $mg^{-1}$, which is in good agreement with the findings of other groups (Denninger et al., 2000; Lee et al., 2001). The present study showed only slight alterations in the affinity of soluble guanylyl cyclase towards its substrate GTP under all test conditions. A small increase in $K_m$ was observed in the presence of DEA/NO, which stands in contrast to findings of other groups (Denninger et al., 2000; Lee et al., 2001). This discrepancy may be due to the very low concentration of DEA/NO (10 nM) that was used in the present study compared to the micromolar amounts applied by other groups (Denninger et al, 2000; Lee et al., 2001). A recently postulated model from Ballou et al. (2002) for a more complex two-step activation of soluble guanylyl cyclase by NO may serve for an explanation. In that study, the authors show that addition of NO to soluble guanylyl cyclase resulted in the rapid formation of a six-coordinated intermediate complex that had no cGMP generating activity until cleavage of the heme–histidin bond in a second slower step. According to the authors, this second soluble guanylyl cyclase activating step is dependent on the NO concentration. Therefore, the very low concentration of NO used in our study may result in a $K_m$ value slightly different than the one found at micromolar concentrations of NO.

Furthermore, a slight increase of $K_m$ for the BAY 41-8543-activated enzyme in the presence of ODQ was observed. However, this increase might be comparable to the increase of $K_m$ found by others for the NO stimulated enzyme in the presence of ODQ (Olsen et al., 1997). The minor changes of $K_m$ in the presence of BAY 41-8543 or BAY 58-2667 were too modest to be responsible for the major part of the soluble guanylyl cyclase activation observed in the activity assay. Therefore, the main part of this activation must be due to the increase in the maximal catalytic rate $V_{max}$.

The different characteristics of the heme-dependent and heme-independent soluble guanylyl cyclase activators found in the activation profile were reflected in the $V_{max}$ values. A strong synergism between BAY 41-8543 and NO for the maximal catalytic rate was observed concomitant with the findings of other groups for YC-1 (Denninger et al., 2000; Lee et al., 2001). A strictly additive effect on $V_{max}$ was found for BAY 58-2667 in combination with NO. In the

presence of ODQ, we observed a decrease of $V_{max}$ with BAY 41-8543-activated soluble guanylyl cyclase but a strong potentiation of $V_{max}$ in the presence of BAY 58-2667. Taken together, these results show clearly that the differences of the heme-dependent and heme-independent soluble guanylyl cyclase activators observed in the activation profile are primary due to the strong increase of the maximal catalytic rate.

In contrast to the synergism observed with the heme-dependent soluble guanylyl cyclase stimulator BAY 41-8543, the activation profile and the maximal catalytic rate of BAY 58-2667-activated soluble guanylyl cyclase showed only an additive effect in combination with NO. Therefore, we reasoned whether the presence of BAY 58-2667 or BAY 41-8543 had any effect on the deactivation of NO-stimulated soluble guanylyl cyclase. We determined the half-life of the nitrosyl–heme complex at the native soluble guanylyl cyclase to be 1.6 s, which is in good agreement with findings of other groups (Kharitonov et al., 1997; Russwurm et al., 2002). According to these authors, YC-1 prolonged this half-life by more than 155-fold. In our study, BAY 41-8543 acted in a similar way by increasing the half-life of heme-bound NO by 220-fold. In contrast, BAY 58-2667 did not alter distinctly the half-life of the nitrosyl–heme complex. These results could explain the observed strong synergism of BAY 41-8543 and NO (Stasch et al., 2002a) whereas the lack of any overadditive effect of BAY 58-2667 and NO on soluble guanylyl cyclase activity could be due to the missing prolongation of the half-life of the nitrosyl–heme complex.

In summary, our results underline that the heme-dependent soluble guanylyl cyclase stimulators and heme-independent soluble guanylyl cyclase activators address two different mechanisms of activating the enzyme. Both classes of compounds show similar characteristics in the spectroscopic studies due to the lack of interaction with the central iron of the prosthetic heme moiety. However, they differ markedly in the soluble guanylyl cyclase activation profile. The activity assay shows a strong synergism of BAY 41-8543 combined with NO that can be explained by stabilization of the nitrosyl–heme complex. This synergism is abolished by ODQ. In contrast, BAY 58-2667 produces a mere additive effect on soluble guanylyl cyclase activation in combination with NO and activates soluble guanylyl cyclase even in the presence of ODQ. These activation profiles are mainly due to the strong increase in the maximal catalytic rate and not to changes in the affinity of soluble guanylyl cyclase towards its substrate GTP. The absence of any synergism of BAY 58-2667 and NO might be a consequence of the inability of this compound to stabilize the nitrosyl–heme complex. Both classes of activators can serve as powerful biochemical tools for the understanding of the complex activation of soluble guanylyl cyclase and, moreover, may offer new approaches for treating cardiovascular diseases.

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