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# Residues stabilizing the heme moiety of the nitric oxide sensor soluble guanylate cyclase

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**Abstract**

Soluble guanylate cyclase, a heterodimer consisting of an α- and a heme-containing β-subunit, is the major receptor for the biological messenger nitric oxide (NO) and is involved in various signal transduction pathways. The heme moiety of the enzyme is bound between the axial heme ligand histidine$_{105}$ and the recently identified counterparts of the heme propionic acids, tyrosine$_{135}$ and arginine$_{139}$. The latter residues together with an invariant serine$_{137}$ form the unique heme binding motif **Y**-x-**S**-x-**R**. In this work, we show that replacement of the serine$_{137}$ with alanine destabilizes the binding of the heme moiety and impairs NO-mediated soluble guanylate cyclase activation.


*Keywords:* Soluble guanylate cyclase; Heme; Nitric oxide; cGMP; BAY 58-2667; BAY 41-2272; YC-1

## 1. Introduction

Soluble guanylate cyclase, the intracellular receptor for the ubiquitous biological messenger nitric oxide (NO), is a heterodimer consisting of an α- and β-subunit. The enzyme contains a prosthetic heme group that is bound via the residues histidine$_{105}$, tyrosine$_{135}$ and arginine$_{139}$ within the β-subunit of soluble guanylate cyclase (Wedel et al., 1994; Zhao et al., 1998; Schmidt et al., 2004). Activation of the enzyme upon binding of its physiological activator NO to the heme moiety catalyzes the conversion of GTP to cGMP. This second messenger regulates various effector systems such as phosphodiesterases, ion channels and protein kinases, thereby modulating many physiological processes including vasodilatation, neurotransmission and platelet aggregation (Hobbs, 2000, 2002). An insufficient soluble guanylate cyclase activation has been identified in various pathological conditions, especially those affecting the cardiovascular system (Moncada et al., 1991; Hobbs, 2000, 2002). For this reason, compounds capable of activating this enzyme in a NO-independent manner represent a promising new therapeutic strategy.

In recent years several NO-independent soluble guanylate cyclase-activators have been identified (Ko et al., 1994; Stasch et al., 2001, 2002a,b). Based upon their characteristics, these compounds can be classified into two groups, the first comprising the NO-independent, but heme-dependent soluble guanylate cyclase-stimulators such as YC-1 (3-(5′-hydroxymethyl-2′-furyl)-1-benzylindazole), BAY 41-2272 and BAY 41-8543 and the second, the NO- and heme-independent soluble guanylate cyclase activators

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represented by BAY 58-2667. The first group shows a strong synergism when combined with NO and a loss of effect after the removal of the prosthetic soluble guanylate cyclase heme moiety (Stasch et al., 2001; Friebe et al., 1996; Schmidt et al., 2003). In contrast, soluble guanylate cyclase activation by compounds like BAY 58-2667 is potentiated by the removal of the heme group due to a high affinity binding site for this compound within the heme pocket of the apo-enzyme (Schmidt et al., 2004; Stasch et al., 2002b). The replacement of the heme group by BAY 58-2667 can be strongly facilitated by oxidation of the heme moiety resulting in a destabilization of the heme binding to the enzyme (Hobbs, 2000; Schmidt et al., 2004).

Recently, we could demonstrate that the complementary heme-dependency of both classes of non-NO soluble guanylate cyclase-activators combined with NO and the heme oxidizing soluble guanylate cyclase-inhibitor ODQ (1H-(1,2,4)-oxadiazole-(4,3-a)-quinoxalin-1-one) allowed a non-invasive intracellular differentiation between heme-free and heme-containing soluble guanylate cyclase. Combined with a novel cGMP reporter cell line, this pharmacological approach allows a fast, easy and reliable screening of mutations that effect the heme-content of soluble guanylate cyclase in an intact cellular environment without the need for further purifications (Schmidt et al., 2004). This approach led to the identification of the residues tyrosine$_{135}$ and arginine$_{139}$ as critically important for the binding of the prosthetic heme moiety. Together with serine$_{137}$, these two amino acids form the unique heme-binding motif **Y**-x-**S**-x-**R** (Fig. 1). In contrast to the central role of tyrosine$_{135}$ and arginine$_{139}$ in coordinating the heme moiety, the role of serine$_{137}$ remained unclear.

To elucidate the role of serine$_{137}$ in the proposed heme pocket we substituted this amino acid with alanine and investigated the influence of this mutation in the cGMP readout cell after incubation with BAY 41-2272, BAY 58-2667, NO and ODQ. In addition, reconstitution studies with PPIX (3,18-divinyl-2,7,13,17-tetramethylporphine-8,12-dipropionic acid) were performed.

## 2. Materials and methods

### 2.1. Compounds

BAY 58-2667 (4-[((4-carboxybutyl){2-[(4-phenethyl-benzyl)oxy]-phenethyl}amino)methyl[benzoic]acid) and BAY 41-2272 (5-cyclopropyl-2-[1-(2-fluoro-benzyl)-1H-pyrazolo[3,4-b]pyridin-3-yl]-pyrimidin-4-ylamine) were synthesized as described (Alonso-Alija et al., 2001; Straub et al., 2001). DEA/NO (2-(*N*,*N*-Diethylamino)-diazenolate-2-oxide), ODQ (1H-(1,2,4)-Oxadiazole-(4,3-a)-quinoxalin-1-one) and PPIX (3,18-Divinyl-2,7,13,17-tetramethylporphine-8,12-dipropionicacid) were purchased from Alexis Biochemicals (San Diego, USA). All other chemicals of analytical grade were obtained from Sigma (Taufkirchen, Germany).

### 2.2. Mutagenesis

The mutagenesis was performed using the QuikChange® site-directed mutagenesis kit (Stratagene, La Jolla, USA) according to the manufacturer's instructions. The following primer was used to perform the βS$_{137}$A mutation: 5′-GCAAAGGGCTCATTCTGCACTACTACGCGGAAA-GAGAGGGGCTTCAGG-3′. The accuracy of the mutation was verified by sequencing (Invitek, Berlin, Germany).

### 2.3. Generation of the cGMP reporter cell

The cGMP reporter cell was generated and cultured as previously described (Schmidt et al., 2004; Wunder et al., 2003).

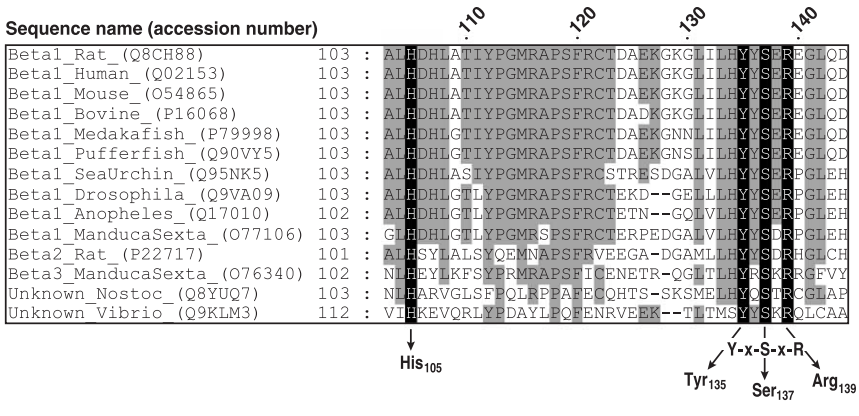


Fig. 1. Multiple sequence alignment of selected amino acid sequences with homology to the N-terminal fragment of the β$_1$-subunit of soluble guanylate cyclase. Sequences were chosen for the alignment which were known or presumed to bind heme. Conserved residues are shaded in grey. Histidine$_{105}$, tyrosine$_{135}$, arginine$_{139}$ and the mutated position serine$_{137}$ are marked by a black background. The name of the sequence is followed by its accession number in brackets.

### 2.4. Transient transfection and cGMP readout

The transient transfection of both soluble guanylate cyclase subunits into the cGMP reporter cells and the subsequent initiation of the cGMP readout was performed as previously described (Schmidt et al., 2004).

### 2.5. Soluble guanylate cyclase-activity assay

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).

### 2.6. PPIX reconstitution

To investigate whether the introduced mutation influenced the heme binding capacity of the enzyme, wildtype (WT)-soluble guanylate cyclase and $\beta S_{137}A$-soluble guanylate cyclase were transiently expressed in chinese hamster ovary (CHO) cells as described above. After 48 h cells were lysed and centrifuged at $100{,}000 \times g$. The soluble guanylate cyclase containing supernatant was applied to the soluble guanylate cyclase activity assay. Reconstitution was performed with increasing concentrations of PPIX in the presence of 0.5% Tween-20 and 1 μM BAY 41-2272 to amplify the PPIX-induced soluble guanylate cyclase activation (Schmidt et al., 2004; Stasch et al., 2002b; Friebe and Koesling, 1998).

## 3. Results

### 3.1. Screening WT- and $\beta S_{137}A$-soluble guanylate cyclase in the cGMP reporter cell

Very recently we could demonstrate that by using NO, BAY 41-2272, BAY 58-2667 and ODQ a differentiation between heme-containing and heme-free soluble guanylate cyclase within an intact cytosolic environment can be achieved (Schmidt et al., 2004). The soluble guanylate cyclase activation by NO and the heme-dependent soluble guanylate cyclase stimulator BAY 41-2272 and also the potentiation of the BAY 58-2667-induced soluble guanylate cyclase activation by the heme-oxidizing soluble guanylate cyclase inhibitor ODQ are clear proofs for the presence of the prosthetic heme moiety. In contrast, the loss of soluble guanylate cyclase activation by NO or BAY 41-2272 and the lack of any potentiation of the BAY 58-2667 induced enzyme activation by ODQ are characteristics of heme-deficient soluble guanylate cyclase. The WT-soluble guanylate cyclase and $\beta S_{137}A$-soluble guanylate cyclase were transiently transfected into a novel cGMP reporter cell line and enzyme activation after incubation with the indicated compounds was measured as relative light units (RLUs) as recently described (Schmidt et al., 2004). Relative light units correlate with intracellular cGMP concentration in stable soluble guanylate cyclase CHO reporter cell line (Wunder et al., 2003, 2005) demonstrated by BAY 41-2272 with $ED_{50}$ values of about 0.1 μM (Fig. 6).

#### 3.1.1. WT-soluble guanylate cyclase

As shown in Fig. 2A–C, CHO cells cotransfected with WT $\alpha_1$- and $\beta_1$-cDNA exhibited the activation profile of heme-containing soluble guanylate cyclase. DEA/NO activated the transiently transfected reporter cells to a maximum of 1,720,000 relative light units with an $EC_{50}$ value of 60 nM (Fig. 2A). In addition, a concentration dependent stimulation of 1,820,000 relative light units could be achieved by the NO-independent, but heme-dependent soluble guanylate cyclase stimulator BAY 41-2272 (Fig. 2B). In the presence of 100 nM DEA/NO the maximal BAY 41-2272 induced enzyme activation was increased to 3,000,000 relative light units. The $EC_{50}$ value for BAY 41-2272 was decreased from 628 nM to 55 nM (Fig. 2B). The NO- and heme-independent soluble guanylate cyclase activator BAY 58-2667 showed a concentration-dependent activation of the transiently transfected reporter cells of 560,000 relative light units, that was potentiated up to 1,200,000 in the presence of the soluble guanylate cyclase inhibitor ODQ (Fig. 2C).

#### 3.1.2. $\beta S_{137}A$-soluble guanylate cyclase

The $\beta S_{137}A$-soluble guanylate cyclase shows an activation profile that differed from the one observed for the WT enzyme. The mutant-soluble guanylate cyclase could be activated by the heme-dependent soluble guanylate cyclase-stimulator BAY 41-2272 to a maximum of 610,000 relative light units that was still potentiated by additional DEA/NO up to 1,000,000 relative light units (Fig. 2E). Both observations indicate the presence of the prosthetic heme moiety, however, the activation was less than that of the WT-soluble guanylate cyclase. The activation of the $\beta S_{137}A$-mutant by DEA/NO was strongly diminished (Fig. 2D). The potentiating effect of ODQ on the BAY 58-2667 activated $\beta S_{137}A$-soluble guanylate cyclase was negligible and increased the maximum activity from 1,230,000 relative light units in the absence of ODQ to 1,400,000 relative light units after addition of ODQ (Fig. 2F).

### 3.2. Enzymatic activity of WT- and $\beta S_{137}A$-soluble guanylate cyclase

CHO cells were transiently transfected with either WT-soluble guanylate cyclase or $\beta S_{137}A$-soluble guanylate cyclase, lysed and the $100{,}000 \times g$ supernatant used for the enzymatic soluble guanylate cyclase-activity assay. The WT-soluble guanylate cyclase lysate displayed the typical activation pattern of heme-containing soluble guanylate cyclase. Incubation with 10 μM DEA/NO resulted in a 119-fold stimulation (Fig. 3). In addition,

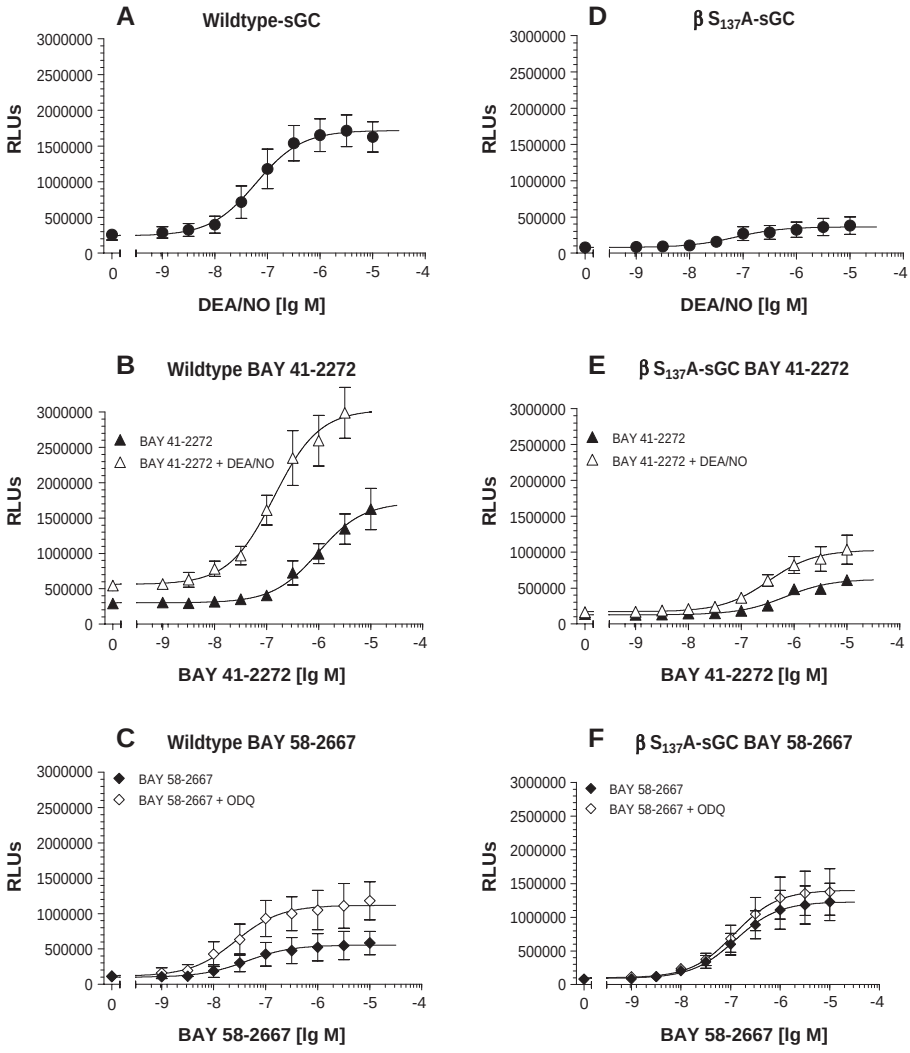


Fig. 2. Activation pattern of WT-soluble guanylate cyclase (A–C) and $\beta S_{137}$A-soluble guanylate cyclase (D–F) incubated with increasing concentrations of the NO donor DEA/NO and the NO independent soluble guanylate cyclase activators BAY 41-2272 or BAY 58-2667 alone or in the presence of a fixed concentration DEA/NO (100 nM) or ODQ (10 μM). cGMP readout cells were transiently cotransfected with the WT $\alpha_1$-subunit and WT or mutated $\beta_1$-subunit of soluble guanylate cyclase. Enzyme activation is represented as relative light units (RLUs). Data are mean ± S.E.M. from 4 to 6 independent experiments performed in quadruple.

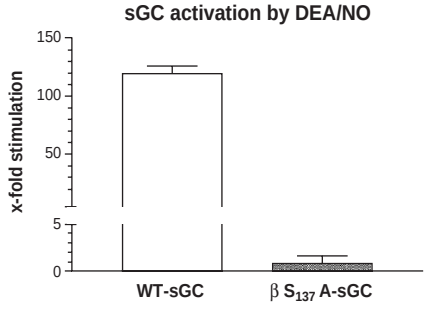


Fig. 3. Activation of the WT-soluble guanylate cyclase or $\beta S_{137}$A-soluble guanylate cyclase containing 100,000 × *g* supernatant after incubation with the NO-donor DEA/NO (10 μM). Data are mean ± S.E.M. from 3 to 4 independent experiments performed in duplicate. Soluble guanylate cyclase activation is represented as *x*-fold compared to the transfected but not stimulated control.

the heme-dependent soluble guanylate cyclase stimulator BAY 41-2272 activated the WT-enzyme to a maximum of 30-fold (Fig. 4A). In the presence of 100 nM DEA/NO, a concentration that results in an soluble guanylate cyclase activation of 43-fold, the maximal activation of BAY 41-2272 was potentiated up to 223-fold (Fig. 4A). Incubation of the WT-enzyme with the heme-independent soluble guanylate cyclase-activator BAY 58-2667 resulted in a maximal soluble guanylate cyclase-activation of 33-fold in the absence and 107-fold in the presence of the heme-oxidizing soluble guanylate cyclase inhibitor ODQ (Fig. 4B).

In contrast to these findings, the $\beta S_{137}$A-soluble guanylate cyclase lysate exhibited an activation profile expected for heme-deficient soluble guanylate cyclase. DEA/NO was

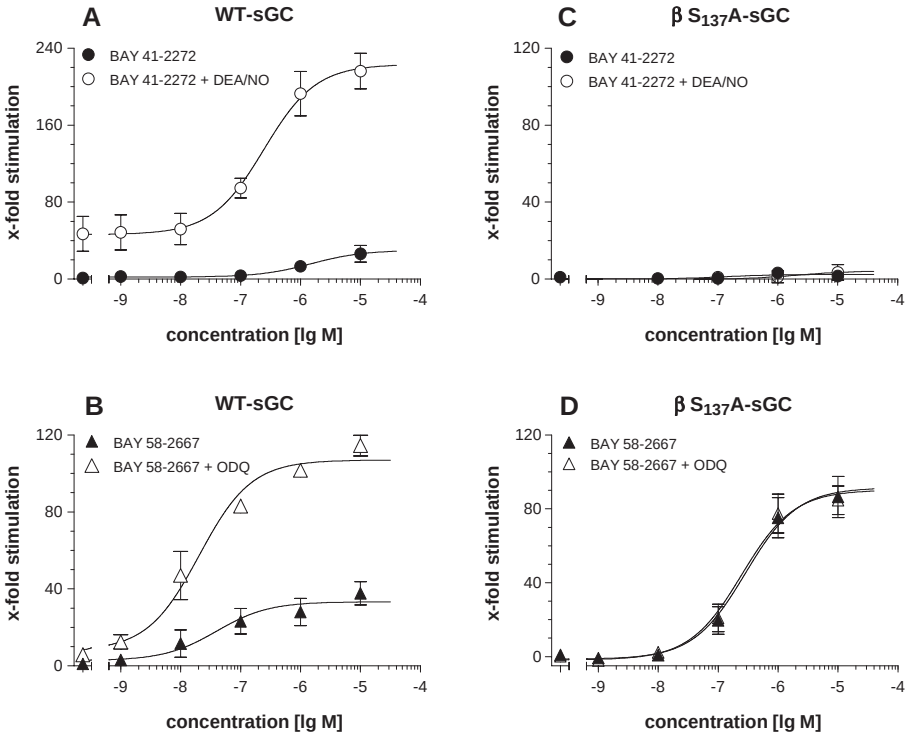


Fig. 4. Concentration response curve of the 100,000 × *g* supernatant containing WT-soluble guanylate cyclase (A, B) or $\beta S_{137}$A-soluble guanylate cyclase (C, D) after incubation with increasing concentrations of the NO independent soluble guanylate cyclase activators BAY 41-2272 and BAY 58-2667 alone or in the presence of a fixed concentration DEA/NO (100 nM) or ODQ (10 μM). Data are mean ± S.E.M. from 3 to 4 independent experiments performed in duplicate. Soluble guanylate cyclase activation is represented as *x*-fold compared to the transfected but not stimulated control.

unable to activate the mutated enzyme even at micromolar concentrations (Fig. 3). The activating effect of the heme-dependent soluble guanylate cyclase-stimulator BAY 41-2272 was completely abolished in the absence as well as in the presence of 100 nM DEA/NO (Fig. 4C). The heme-independent soluble guanylate cyclase-activator BAY 58-2667 activated the mutated soluble guanylate cyclase in a concentration-dependent manner up to 91-fold (Fig. 4D). However, this maximal activation was not further increased upon addition of ODQ (Fig. 4D).

### *3.3. PPIX reconstitution of WT- and $\beta S_{137}$A-soluble guanylate cyclase*

To explore the effect of the $\beta S_{137}$A mutation upon porphyrin binding, the enzyme containing 100,000 × *g* supernatant of transiently transfected and harvested CHO cells was used for PPIX reconstitution studies. PPIX mimics the nitrosyl-heme complex of the NO-activated enzyme (Friebe and Koesling, 1998; Ignarro et al., 1982) and represents therefore a useful tool to investigate the progress

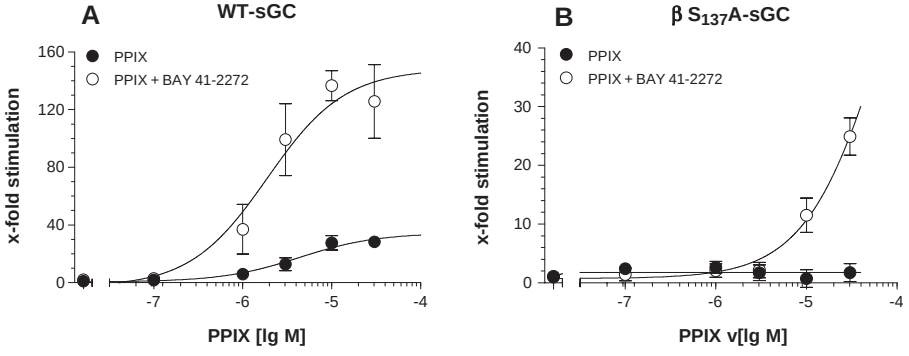


Fig. 5. Concentration response curve of the 100,000 × *g* supernatant containing WT-soluble guanylate cyclase (A) or $\beta S_{137}$A-soluble guanylate cyclase (B) after incubation with increasing concentrations of PPIX in the absence and presence of BAY 41-2272 (1 μM). Assays were performed in the presence of Tween-20 (0.5%). Data are mean ± S.E.M. from 3 to 5 independent experiments performed in duplicate. Soluble guanylate cyclase activation is represented as *x*-fold compared to the transfected but not stimulated control.

of soluble guanylate cyclase reconstitution with increasing concentrations of PPIX (Stasch et al., 2002a; Friebe and Koesling, 1998; Ignarro et al., 1982, 1984). Reconstitution was performed in the presence of Tween-20 for both the removal of the native heme moiety and to facilitate the subsequent reconstitution with PPIX (Foerster et al., 1996). Furthermore, the assay was performed in the absence and presence of BAY 41-2272. Due to its heme-dependency this compound served as an intrinsic control for the progress of the PPIX reconstitution. (Stasch et al., 2002a; Friebe and Koesling, 1998).

WT-soluble guanylate cyclase could be reconstituted with increasing concentrations of PPIX leading to a maximal soluble guanylate cyclase activation of 34-fold in the absence and 148-fold in the presence of the heme-dependent soluble guanylate cyclase stimulator BAY 41-2272 (Fig. 5A). The $EC_{50}$ value for PPIX was shifted from 4.3 μM to 1.9 μM after addition of BAY 41-2272. $\beta S_{137}A$-soluble guanylate cyclase could not be activated by PPIX even at micromolar concentrations (Fig. 5B). However, incubation with 30 μM PPIX in the presence of BAY 41-2272 resulted in an enzyme activation of 25-fold, indicating successful PPIX-reconstitution of the mutant-enzyme (Fig. 5B).

## 4. Discussion

Recently, we were able to differentiate for the first time between heme-free and heme-containing soluble guanylate cyclase using the complementary heme-dependency of both classes of non-NO soluble guanylate cyclase-activators, exemplified by BAY 41-2272 and BAY 58-2667, in combination with a novel cGMP reporter cell line (Schmidt et al., 2004). Hereby cGMP production is monitored in living cells on the basis of luminescence, indicated in relative light units, which correlates directly with the intracellular cGMP level as recently described (Fig. 6; Wunder et al., 2005). This methodical approach made it possible to investigate mutations that effect the heme binding capability of soluble guanylate cyclase in an intracellular environment, eliminating the problem of artificial heme loss caused by the purification procedure (Hoenicka et al., 1999). Due to the above-mentioned technique, we were able to identify the unique soluble guanylate cyclase heme-binding motif **Y**-x-**S**-x-**R** and to prove the importance of $tyrosine_{135}$ and $arginine_{139}$ for the binding of the heme moiety to soluble guanylate cyclase (Schmidt et al., 2004; Karow et al., 2004). Very recently, these findings and therefore the pharmacological approach of the intracellular determination of the soluble guanylate cyclase heme content were confirmed by crystallization studies with prokaryotic protein that is sequence related to soluble guanylate cyclases (Pellicena et al., 2004). The crystal structure indicates a role for the $serine_{137}$ in the binding of the heme moiety, however, its influence for the NO-independent activation of soluble guanylate cyclase remained unclear. In this report we investigated the contribution of this amino acid for the binding of the prosthetic heme moiety and the NO-independent activation of soluble guanylate cyclase. We could show that the exchange of this serine to alanine destabilizes the binding of the heme group to the apo-enzyme. However, this residue appears to be less important for the coordination of the heme moiety than the $tyrosine_{135}$ and $arginine_{139}$ previously described (Schmidt et al., 2004).

Within the cellular cGMP reporter system, incubation with the heme-dependent soluble guanylate cyclase-stimulator BAY 41-2272 resulted in the typical activation pattern for heme containing soluble guanylate cyclase for both the WT-enzyme and the $\beta S_{137}A$-mutant. Addition of DEA/NO potentiated the BAY 41-2272 induced enzyme activity of the WT-soluble guanylate cyclase. This is in agreement with findings reported earlier for intracellular expressed and purified heme-containing WT-soluble guanylate cyclase (Schmidt et al., 2004; Stasch et al., 2001). The observed reduction in activation might be a hint for a possible involvement of $serine_{137}$ in the intramolecular signal transmission. These results indicate that the $\beta S_{137}A$-soluble guanylate cyclase was expressed as a heme-containing enzyme with comparable characteristics to that of the WT-enzyme, but with an impaired response for both BAY 41-2272 and DEA/NO.

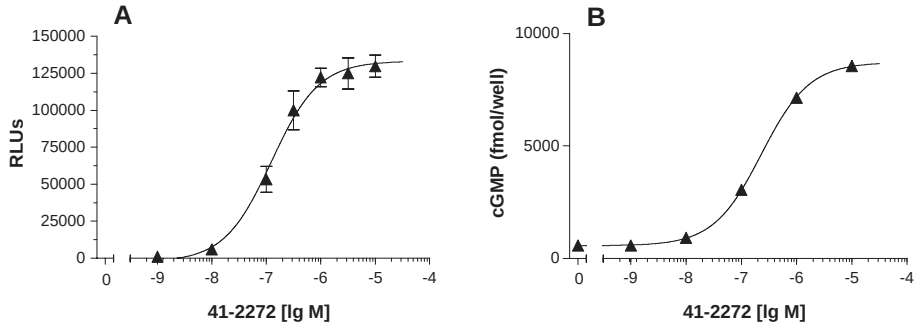


Fig. 6. Effects of BAY 41-2272 on soluble guanylate cyclase activity in the stable recombinant cell line. (A) Stimulation of the luminescence signal by increasing concentrations of BAY 41-2272. (B) Concentration-dependent intracellular cGMP formation in confluent 24-well plates of soluble guanylate cyclase overexpressing CHO cells measured by radioimmunoassay. Data are presented as means ± S.E.M.

The $\beta S_{137}A$-soluble guanylate cyclase containing $100{,}000 \times g$ supernatant of the transiently transfected and harvested cells showed the activation pattern of heme deficient soluble guanylate cyclase. Neither NO, the heme-dependent soluble guanylate cyclase stimulator BAY 41-2272, nor the combination of both resulted in an activation of $\beta S_{137}A$-soluble guanylate cyclase. In addition, ODQ, which also depends upon the presence of the heme group, failed to potentiate the BAY 58-2667 induced activation of the mutant soluble guanylate cyclase. Both observations indicate unequivocally the absence of the heme moiety in contrast to the results obtained from the cellular cGMP reporter system discussed above. This can be explained by a strong reduction in the affinity of the enzyme for the prosthetic heme group due to the induced mutation. Therefore, lysing the transiently transfected cells resulted in the loss of the weakly bound heme moiety. This view is also supported by the observed negligible potentiation of the BAY 58-2667 induced activation of $\beta S_{137}A$-soluble guanylate cyclase by ODQ within the cellular system. The oxidation of the heme by ODQ facilitates its replacement by BAY 58-2667 due to a destabilization of the heme soluble guanylate cyclase bond (Schmidt et al., 2004; Hobbs, 2000). In the case of the mutation-induced reduction in heme affinity, even the reduced heme could be easily displaced by BAY 58-2667. Therefore, additional ODQ would have no further potentiating effect upon the BAY 58-2667 induced activation of the mutant soluble guanylate cyclase. In addition, the reduced affinity of the heme group resulted in higher concentrations of PPIX required for the reconstitution of $\beta S_{137}A$-soluble guanylate cyclase compared to the WT-enzyme. Taken together, this data points to a heavily reduced affinity of the enzyme for its heme-ligand.

Indeed, optical absorption spectra of the WT and mutant soluble guanylate cyclase would be the direct way to show the presence or absence of the prosthetic heme moiety of the enzyme. The measurement of the native heme spectra requires expressed and highly purified enzyme. However, the purification of soluble guanylate cyclase from cells is, as published by various groups, a time consuming and critical step due to the complicated purification procedure. They reported a loss of heme during this purification procedure that was independent from mutational alteration of the soluble guanylate cyclase (Hoenicka et al., 1999). Therefore, this approach may also lead to artificial heme loss, which allows no conclusion regarding the intracellular state of the enzyme to be drawn. In contrast to these obstacles, the heme-independent soluble guanylate cyclase-activator, BAY 58-2667 allows for the first time a measurement of the soluble guanylate cyclase-heme content within an intracellular environment.

In agreement with the impaired synergism of BAY 41-2272 and DEA/NO observed in the cellular reporter system for the $\beta S_{137}A$-soluble guanylate cyclase, the activation of this mutant-soluble guanylate cyclase by DEA/NO was also strongly impaired. This observation is further supported by the PPIX reconstitution studies. Although the $\beta S_{137}A$-soluble guanylate cyclase was successfully reconstituted with PPIX, as indicated by the increasing enzyme activity in the presence of the heme-dependent soluble guanylate cyclase stimulator BAY 41-2272, PPIX alone failed to activate the mutant-soluble guanylate cyclase. Since PPIX mimics the nitrosyl-heme complex (Friebe and Koesling, 1998; Ignarro et al., 1982, 1984), these results suggest an impairment of the intramolecular signal transmission upon NO binding to the heme moiety. In summary, we show that the exchange of $serine_{137}$ within the soluble guanylate cyclase heme binding motif **Y**-x-**S**-x-**R** to alanine destabilizes binding of the heme moiety to the enzyme.

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