**NO-independent regulatory site of direct sGC stimulators like YC-1 and BAY 41-2272**

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**Abstract**

**Background:** The most important receptor for nitric oxide is the soluble guanylate cyclase (sGC), a heme containing heterodimer. Recently, a pyrazolopyridine derivative BAY 41-2272, structurally related to YC-1, was identified stimulating soluble guanylate cyclase in an NO-independent manner, which results in vasodilatation and antiplatelet activity. The study described here addresses the identification of the NO-independent site on soluble guanylate cyclase.

**Results:** We developed a photoaffinity label (³H-meta-PAL) for the direct and NO-independent soluble guanylate cyclase (sGC) stimulator BAY 41-2272 by introducing an azido-group into the tritium labeled compound. The synthesized photoaffinity label directly stimulates the purified sGC and shows in combination with NO a synergistic effect on sGC activity. Irradiation with UV light of ³H-meta-PAL together with the highly purified sGC leads to a covalent binding to the α₁-subunit of the enzyme. This binding is blocked by unlabeled meta-PAL, YC-1 and BAY 41-2272. For further identification of the NO-independent regulatory site the ³H-meta-PAL labeled sGC was fragmented by CNBr digest. The ³H-meta-PAL binds to a CNBr fragment, consisting of the amino acids 236–290 of the α₁-subunit. Determination of radioactivity of the single PTH-cycles from the sequencing of this CNBr fragment detected the cysteines 238 and 243 as binding residues of the ³H-meta-PAL.

**Conclusions:** Our data demonstrate that the region surrounding the cysteines 238 and 243 in the α₁-subunit of the sGC could play an important role in regulation of sGC activity and could be the target of this new type of sGC stimulators.

**Background**

Guanulate cyclases (GTP pyrophosphate-lyase [cyclizing], EC 4.6.1.2) catalyze the biosynthesis of guanosine 3',5'-cyclic monophosphate (cGMP) from GTP. While the
membrane bound forms are monomers and stimulated by the natriuretic peptides, the soluble guanylate cyclases (sGC) exist as heterodimers consisting of an α₃- and a β₁-subunit and containing heme as a prosthetic group [1]. Besides this major species of sGC there exist also reports of homodimeric forms of this enzyme [2,3]. By formation of cGMP as a second messenger, sGC plays an important role in smooth muscle cell relaxation [4], inhibition of platelet aggregation, retinal signal transduction [5] and synaptic transmission [6]. The enzyme is strongly activated by NO [7], by the new direct and NO-independent stimulator YC-1 [8–11], and to a lesser extend by CO [12–14,11]. Thus in several studies YC-1 was shown to be an antithrombotic agent by elevation of cGMP, VASP phosphorylation and inhibiting platelet aggregation [8,13,15–18]. Furthermore YC-1 was shown to relax precontracted aortic rings [9], even if they were made tolerant with glyceryl trinitrite [19] and to induce a dose-dependent decrease in systolic blood pressure [19]. Interestingly, in addition to the direct activation of the purified sGC by YC-1, an overadditive effect was observed by the combinations of YC-1 and NO or CO [9–11,18,20]. It was shown that YC-1 is a heme-dependent but NO-independent stimulator of sGC [11,13]. In contrast to NO and CO which directly interact with the heme group of the enzyme, YC-1 did not change the spectral characteristics of the heme moiety of sGC [11]. Therefore it is believed that YC-1 did not interact directly with the heme region of the sGC. Because YC-1 sensitises the enzyme towards their endogenous ligands NO and CO, it is proposed that YC-1 binds to an allosteric site on sGC and thereby reduces the ligand dissociation rates from the heme group [10,11,20,21].

Recently, we described the pharmacological profile of a new sGC stimulator – BAY 41-2272 with similar characteristics like YC-1, however, with a distinctly higher potency and no PDE inhibitory activity [22]. Using BAY 41-2272 as lead structure of this new pharmacological principle, we synthesized the first photoaffinity label derived from the BAY 41-2272 chemical core structure, characterized it on the purified enzyme, labeled the highly purified sGC, and identified the binding amino acids of this new type of sGC stimulators.

**Results**

We developed the first photoaffinity label for direct and NO-independent sGC stimulators. Coming from YC-1 and BAY 41-2272 as lead structures of direct and NO-independent sGC stimulators we synthesized the ortho- (BAY 50-6038), meta- (BAY 51-9491) and para-PAL (BAY 50-8364) compounds (Fig. 1) and tested their influence on sGC activity. The meta- and para-PAL showed on the one hand a direct sGC stimulation comparable to YC-1 and on the other hand in combination with NO distinct synergistic effects on sGC activity. The ortho-PAL showed the lowest sGC stimulation and in combination with NO no synergistic effect on sGC activity (Tab. 1). Both under reducing conditions and in the absence of DTT, in the sGC assay a similar sGC activating profile of the three PALs is given (Tab. 1). Because of the more dominant stimulation of sGC by the single compound both in the presence and absence of DTT the meta-PAL compound was chosen as photoaffinity label for the following studies (Tab. 1).

Similar to YC-1 and BAY 41-2272, meta-PAL (BAY 51-9491) concentration-dependently stimulates purified sGC and shows in combination with NO a potentiation of sGC activity, while in combination with YC-1, only additive effects could be detected. sGC stimulation by meta-PAL could be nearly completely blocked by ODQ and is dependent of the presence of the prosthetic heme-moiety of sGC (Fig. 3a). As YC-1, meta-PAL does not shift the position of the Soret band of sGC both under basal and NO stimulated conditions (Fig. 3b).

To optimize the irradiation conditions for the following photoaffinity studies, the NH- and CH-insertion qualities of meta-PAL (BAY 51-9491) were studied in the presence of different solution compounds by irradiation under 254 or 365 nm. As shown in Tab. 2, NH-insertion was more effective than CH-insertion under our conditions. UV 254 nm was chosen as irradiation source. In addition, the time dependency of the photoactivation of the meta-PAL (BAY 51-9491) under UV 254 nm (40 Watt, distance of 3 cm) was studied by thin layer chromatography (RF of meta-PAL = 0.7 and of photolyzed meta-PAL = 0.5). The meta-PAL was completely destroyed after irradiation in PAL...
Table 1: Stimulation of purified sGC in the presence and absence of DTT by the NO donor SNP. YC-1 and the ortho-, meta- and para-PALs with Mg²⁺ as cofactor. Shown are the x-fold stimulations versus basal specific activity of SNP (1 μM), YC-1 (100 μM, 10 μM, 1 μM and 1 μM YC-1 in combination with 1 μM SNP) and the different PALs (100 μM, 10 μM, 1 μM and 1 μM PAL in combination with 1 μM SNP). The data presented represent the mean ± SEM out of four independent experiments performed in duplicate.

| Stimulator          | Concentration | + DTT       | -DTT        |
|---------------------|---------------|-------------|-------------|
| Basal               | 1.0 ± 0.1 (64 ± 8) | 1.0 ± 0.2 (150.8 ± 33.4) |
| SNP                 | 1 μM          | 82.3 ± 10.5 | 17.1 ± 2.2  |
|                     | 100 μM        | 45.9 ± 7.7  | 44.4 ± 4.8  |
| YC-1                | 10 μM         | 4.5 ± 0.8   | 7.0 ± 1.7   |
|                     | 1 μM          | 2.0 ± 0.4   | 1.5 ± 0.2   |
| YC-1 + SNP          | 1 μM + 1 μM   | 98.4 ± 17.3 | 20.6 ± 3.6  |
|                     | 100 μM        | 14.4 ± 1.6  | 21.4 ± 4.2  |
| ortho-PAL           | 10 μM         | 2.9 ± 1.5   | 3.1 ± 0.6   |
|                     | 1 μM          | 2.1 ± 1.0   | 1.0 ± 0.1   |
| ortho-PAL + SNP     | 1 μM + 1 μM   | 85.7 ± 11.4 | 15.8 ± 2.0  |
|                     | 100 μM        | 54.7 ± 10.2 | 56.1 ± 0.1  |
| meta-PAL            | 10 μM         | 21.3 ± 7.1  | 23.3 ± 4.1  |
|                     | 1 μM          | 4.5 ± 1.0   | 4.9 ± 0.4   |
| meta-PAL + SNP      | 1 μM + 1 μM   | 185.6 ± 72.6| 39.0 ± 5.6  |
|                     | 100 μM        | 36.6 ± 3.5  | 30.2 ± 4.5  |
| para-PAL            | 10 μM         | 15.3 ± 2.7  | 17.3 ± 1.9  |
|                     | 1 μM          | 3.5 ± 0.4   | 3.8 ± 0.3   |
| para-PAL + SNP      | 1 μM + 1 μM   | 229.1 ± 33.6| 50.6 ± 5.5  |

Buffer within 3 min. In the presence of glucose oxidase as model protein, meta-PAL was completely destroyed after an irradiation time of about 18 min (data not shown). Therefore the irradiation time for the labeling studies was chosen with 15 min under 254 nm (40 Watt) at a distance of 3 cm.

For enzyme labeling, ³H-meta-PAL was synthesized as described (Fig. 2). The binding of ³H-meta-PAL to highly purified sGC was studied both in the presence of different concentrations of sGC as different concentrations of ³H-meta-PAL, and also in the presence of unlabeled meta-PAL (100 μM). As shown in Fig. 4, ³H-meta-PAL binds concentration-dependently almost exclusively to the α₁-subunit of the sGC after irradiation. The radiolabeling of the β₁-subunit is only weakly visible in the autoradiogram after reaction of sGC with 200 μCi ³H-meta-PAL. In the presence of unlabeled meta-PAL (100 μM) the covalent binding of the ³H-meta-PAL to the α₁-subunit is obviously diminished in the presence of 20 or 2 μCi ³H-meta-PAL.

In addition, we examined the effects of different sGC modulators on binding of ³H-meta-PAL on purified sGC (Fig. 5). Both unlabeled meta-PAL (100 μM and 10 μM), YC-1 (100 μM), BAY 41-2272 [22] but also ODQ (100 μM) diminished concentration-dependently the binding of ³H-meta-PAL to the α₁-subunit. ODQ inhibit the binding of the ³H-meta-PAL to the α₁-subunit, but in this case weak unspecific labeling of the β₁-subunit and the α₁-subunit was detected. ³H-meta-PAL processed with sGC without irradiation showed no insertion of radioactivity (Fig. 5).

To identify the binding residues of the ³H-meta-PAL at the α₁-subunit, sGC was labeled with ³H-meta-PAL and the labeled protein was fragmented by CNBr cleavage. The resulting protein fragments were separated by electrophoresis (10–20% SDS-PAGE), transferred to a PVDF membrane, stained with Coomassie-blue and exposed to Imaging-Plates (Fig. 6). The resulted autoradiogram shows five highly labeled protein fragments (CNBr I-V) which were identified and excised from the same fragments were separated on a TRIS-Tricine-gel. In this autoradiogram we detected three highly labeled protein fragments were separated by electrophoresis (10–20% SDS-PAGE), transferred to a PVDF membrane, stained with Coomassie-blue and exposed to Imaging-Plates (Fig. 6). The resulted autoradiogram shows five highly labeled protein fragments (CNBr I-V) which were identified and excised from the same fragments were separated on a TRIS-Tricine-gel.
of sGC were sequenced. We observed, that the first 20 coded amino acids of the \( \alpha_1 \)-subunit probably belong to the presequence. The \( \beta_1 \)-subunit corresponded to the published sequence. Because of the selective labeling of the \( \alpha_1 \)-subunit by the \( 3^H \)-meta-PAL (Fig. 4 and 5), in Tab. 3 only the theoretically CNBr fragments of the \( \alpha_1 \)-subunit (AS 20-690) are aligned and signed after their position. It is known that CNBr fragments show different migration properties in SDS-PAGE related to the used molecular weight markers. Therefore in the figures we used the calculated molecular weights from sequencing to mark the labeled CNBr fragments. The sequenced bands are marked in Fig. 6 and 7. The results of the sequencing of the different fragments are shown in Tab. 4. The overview of the determined \( \alpha_1 \)-sequences shows, that the \( 3^H \)-meta-PAL specifically binds to the CNBr VIII = CNBr-2 fragment of the \( \alpha_1 \)-subunit (Tab. 3 and 4) with the amino acids 236–290.

To more closely specify the binding site of the \( 3^H \)-meta-PAL, the single PTH-cycles from the sequencing of the CNBr VIII fragment (Fig. 7 and Tab. 4) were collected, lyophilised and the radioactivity of each fraction was counted. Beside an unspecific "wash out", we determined a distinct increase of radioactivity in the cycles 3 and 8 (> 10-fold over basal measured activity), which correspond to the cysteines 238 and 243 (Fig. 8). Thereby \( 3^H \)-meta-PAL is bound to sGC after irradiation by the cysteines 238 and 243 of the \( \alpha_1 \)-subunit of the sGC (Fig. 9).

The putative target sequence of bovine sGC differs from rat and human by the substitution of an arginine at position 238 instead of cysteine. Therefore we investigated if BAY 41-2272 is active against the bovine enzyme or in isoleated bovine vascular preparations. We prepared crude sGC containing fraction from bovine lung by performing the homogenization and ion-exchange steps of the method described earlier [11] with a specific basal activity of 0.15 nmol/mg/min. Using this preparation we examined the characteristic of sGC stimulation by BAY 41-2272 alone and in the presence of high concentrations of DEA/NO (Fig. 10). This study shows that BAY 41-2272 stimulates directly the crude sGC-containing fraction from bovine lung in a concentration dependent manner. In combination, BAY 41-2272 and the NO donor DEA/NO potentiates over a wide range of concentrations.
Table 2: CH- and NH-insertion of meta-PAL dissolved in the different solvents after quantitative LC/MS (results are given as % peak area of insertion product vs. total peak area).

| solvent     | NH-insertion | CH-insertion |
|-------------|--------------|--------------|
|             | irradiation wavelength | irradiation wavelength |
|             | 254 nm | 365 nm | 254 nm | 365 nm |
| diethylamine| 87%, 91% | 32%, 23% | - | - |
| dipropylamine| 81%, 91% | 21%, 18% | - | - |
| piperidine  | 61% | 26% | - | - |
| toluene     | - | - | 49% | - |
| ethylbenzole| - | - | 8% | 2% |

Table 3: Sequence of the marked CNBr fragments in figures 5 and 6. MW: molecular weight; AA: amino acid; the several amino acids are shown in the one-letter-code. Sequenced fragments of the α₁-subunit are signed as in Tab. 4, while CNBr fragments of the β₁-subunit are defined by the position of the determined AA in the subunit. The CNBr-2 fragment of the α₁-subunit, represented in all labeled bands.

| CNBr-fragment | α₁-subunit | β₁-subunit | Sequence from the N-terminus | MW [kDa] |
|---------------|------------|------------|-------------------------------|---------|
| CNBr I        | CNBr -1 to 8 | APGQVTEPIEEE... | 51.8 |
| CNBr II       | CNBr -1 to 4 | APGQVTEPIEEE... | 35.9 |
| CNBr III      | CNBr -1 and 2 | APGQVTEPIEEE... | 30.2 |
| CNBr IV       | CNBr 16      | PRYCLGNNVT... | 11.2 |
| CNBr 8        | CNBr -2 and 3 | IYIVESSAILFLG... | 12.5 |
| CNBr 8        | CNBr 8      | PPCFRSECTEF... | 11.7 |
| CNBr V        | CNBr -10 to 13 | EIAQGQVQVDGE... | 12.8 |
| CNBr V        | CNBr 3      | LDRDLAILQ... | 5.5 |
| CNBr V        | CNBr 2      | PPCFRSECTEF... | 6.2 |
| CNBr V        | AA 505-619  | YGFVNHALED... | 7.7 |
| CNBr V        | AA 2-69     | PRYCLFNGTVN... | 6.2 |
| CNBr VI       | CNBr -2 to 6 | PPCFRSECTEF... | 14.7 |
| CNBr VI       | CNBr 16     | PRYCLFNGNV... | 11.2 |
| CNBr VI       | AA 505-599  | EIAQGQVQVDGE... | 10.4 |
| CNBr VII      | CNBr -2 and 3 | PPCFRSECTEF... | 11.7 |
| CNBr VII      | CNBr 8      | IYIVESSAILFLG... | 12.5 |
| CNBr VII      | CNBr 2      | PPCFRSECTEF... | 6.2 |
| CNBr VIII     | CNBr 8      | KIVNLLNDLYTR... | 4.3 |
| CNBr VIII     | AA 445-480  | YGFVNHALED... | 7.7 |
| CNBr VIII     | AA 2-69     | YGFVNHALED... | 7.7 |
| CNBr VIII     | AA 1-69     | YGFVNHALED... | 7.7 |
Discussion

To identify the binding site of the new class of direct and NO-independent sGC stimulators like YC-1 and the recently described more potent compound BAY 41-2272 [22] we focus in this paper in addition to results, already shown in Stasch et al. [22], on the detailed description of the development of the first photoaffinity label by introducing an azidobenzoic acid at different positions (ortho-, meta-, and para-PAL) into the BAY 41-2272 core structure. As shown, all tested photoaffinity labels stimulate the purified enzyme with similar characteristics as YC-1. sGC activity increases from the ortho- to the para- to the meta-PAL compound. In contrast to the ortho-PAL compound, the meta- and para-PAL compounds show synergistic effects on sGC activity in combination with NO as described for YC-1 and BAY 41-2272 [11,22]. Under these conditions meta-PAL reveals to be the most potent compound with the same characteristics on stimulation of sGC as YC-1 and BAY 41-2272. As expected, meta-PAL shows no influence on the position of the Soret band of sGC both under basal and NO-stimulated conditions. Therefore we conclude from these structure-activity-relationships, that the meta- and para-PALs in contrast to the ortho-PAL match structurally with the new binding site of the sGC, essentially regarding to the synergistic activation of sGC in combination with its physiological stimulator NO and thereby are useful tools in the identification of the new binding site in sGC. In addition to Stasch et al.[22], we demonstrate here different potential PALs, which differ in their chemical structure and sGC stimulation characteristics.

In forward to avoid an unproductive destruction of the PAL during the labeling of the enzyme [34], stimulation of sGC was not only tested under standardized conditions [11] but also verified under modified conditions in the absence of DTT. Even under this adapted conditions used for the labeling of the enzyme, the PAL compounds stimulate the purified enzyme with similar properties as YC-1 and BAY 41-2272. Under these conditions meta-PAL reveals to be the most potent compound with the same characteristics on stimulation of sGC as YC-1 and BAY 41-2272. As expected, meta-PAL shows no influence on the position of the Soret band of sGC both under basal and NO-stimulated conditions. Thereby meta-PAL belongs to the new class of direct, NO-independent sGC stimulators, which stimulate sGC by a heme-dependent mechanism without a direct interaction with the heme-moiety [11,22].

Moreover we characterize the insertion characteristics of the meta-PAL. Concomitant with the literature, our meta-PAL showed more potent NH- than CH-insertion qualities and thereby is qualified for protein conjugation reactions [35]. During irradiation with UV light (254 nm), under our adapted conditions the meta-PAL completely disintegrated within a time of maximal 18 min under release of nitrogen, detected by thin-layer-chromatography. Beyond the fast reaction of azido compounds after irradiation with UV, the quality of a photoaffinity label depends on the stability of the compound and the radioactive label and of the covalent binding properties of the compound at or at least in the environment of the binding site [35]. Our compound is stable for several weeks stored at 70°C (data not shown). Moreover, under reducing conditions, where the sGC is distinctly stimulated by the meta-PAL, and thereby the active conformation of the sGC should be

| CNBr fragment | MW [kDa] | AA number | AA position in the α1 subunit | Sequence from the N-terminus |
|---------------|---------|-----------|-------------------------------|-----------------------------|
| CNBr-1        | 24      | 215       | 21–235                        | APGVPTPEIEE...               |
| CNBr-2        | 6.2     | 54        | 236–290                       | PPCFRSCTEFV...               |
| CNBr-3        | 5.5     | 47        | 291–338                       | LDRDLAILQLG...               |
| CNBr-4        | 0.2     | 2         | 339–340                       | TM                           |
| CNBr-5        | 0.3     | 3         | 341–343                       | LNM                          |
| CNBr-6        | 2.5     | 19        | 344–363                       | QFVIRVRWDL...                |
| CNBr-7        | 0.6     | 6         | 364–369                       | DLGQGM                       |
| CNBr-8        | 12.5    | 111       | 370–481                       | IYVESSAFLFL...               |
| CNBr-9        | 2.4     | 22        | 482–504                       | LSFLDIVGTAICS...             |
| CNBr-10       | 5.2     | 46        | 505–551                       | LNALYTRFDOQ...               |
| CNBr-11       | 0.4     | 4         | 552–555                       | ALKM                         |
| CNBr-12       | 0.1     | 1         | 556                           | M                            |
| CNBr-13       | 0.8     | 7         | 557–563                       | ELsvEM                       |
| CNBr-14       | 0.9     | 9         | 564–572                       | SphGEPIM                     |
| CNBr-15       | 1.8     | 17        | 573–590                       | RIGLHGSFVAG...               |
| CNBr-16       | 11.2    | 99        | 591–690                       | PRYCLFGNNVVT...               |

Table 4: Theoretical CNBr fragments of the α1-subunit of the sGC. MW: molecular weight; AA: amino acid; the several amino acids are shown in the one-letter-code.
provided, the $^3$H-meta-PAL binds not only concentration-dependently but also specifically to the $\alpha_1$-subunit of the sGC. Ideally, a classical receptor binding study would be the best approach to characterize the specific as well as unspecific binding to sGC. However, due to the physicochemical properties of the radioligand (e.g. low solubility, lipophilic character, low specific activity) and the difficulty that the receptor sGC is a soluble cytosolic enzyme, it was not possible to separate free and bound radioligand. For this reason we were not able to establish this study. Binding to the $\alpha_1$-subunit of the enzyme was inert versus all used analytical steps (e.g. TCA precipitation, acid CNBr proteolysis, SDS-PAGE) and thereby underlines the quality of this new compound.

In a further study, the inhibition of this specifically binding of the $^3$H-meta-PAL to the $\alpha_1$-subunit of the sGC by several sGC modulators was examined. Thereby we could show that the $^3$H-meta-PAL binds only after irradiation to the $\alpha_1$-subunit of the sGC, implicating that the $^3$H-meta-PAL binds as a consequence of irradiation and not by an unspecific interaction to the enzyme. After preincubation with the purified sGC, the unlabeled meta-PAL(10 and 100 $\mu$M) as well as YC-1 (100 $\mu$M) or BAY 41-2272 inhibited the insertion of the $^3$H-meta-PAL in the $\alpha_1$-subunit, underlining the specificity of the binding. As shown in Fig. 4, incubation with 200 $\mu$Ci (125 $\mu$M) $^3$H-meta-PAL in the presence of 100 $\mu$M unlabeled PAL showed only slight competition due to the small access of unlabeled compound. For this reason further competition studies were performed with 12.5 $\mu$M $^3$H-meta-PAL (Fig. 5). In addition, the specific inhibitor of sGC, ODQ [36], strongly diminished the binding of the $^3$H-meta-PAL to the $\alpha_1$-subunit. In this case there could be detected an equivalent binding of the $^3$H-meta-PAL both to the $\alpha_1$- and the $\beta_1$-subunit. It is described that ODQ inhibits sGC activity by oxidation of the heme moiety from Fe$^{2+}$ to Fe$^{3+}$ or by competition with the NO binding site [37,38]. Therefore we propose that after interaction with ODQ the native heme environment of the sGC is changed (e.g. by oxidation), destroying the binding site for direct and NO-independent sGC stimulators and therefore $^3$H-meta-PAL unspecifically binds to both subunits of the sGC. The slightly stronger labeling of the beta subunit would result in this context from the possible higher available concentration of free meta-PAL. This explanation is underlined by the observation, that the heme-depleted enzyme is insensitive towards stimulation by meta-PAL and other direct and NO-independent sGC stimulators like BAY 41-2272 or YC-1 [13,22]. Recently, Martin et al. [46] showed that the stimulation of sGC by the new type of sGC stimulators like YC-1 has both heme-dependent and heme-independent components. The stimulation of sGC by YC-1 is not completely blocked by ODQ, in particular in higher concentrations of sGC stimulators. They conclude, that the binding of ODQ and YC-1 are not mutually exclusive processes and that their binding sites do not overlap. These data corroborate our findings.

In addition, the $^3$H-meta-PAL labeled sGC was fragmented by CNBr digest to identify the binding site of the $^3$H-PAL at the $\alpha_1$-subunit more closely. In this study, the chemical digest of the labeled sGC shows five highly labeled protein fragments on the Photo-Imaging-Plate of the gradient gel in the molecular range between 6.2 and 9.0 $\mu$g. These data corroborate our findings.

![Figure 4](image-url)

**Figure 4**
Autoradiogram of $^3$H-meta-PAL (2, 20 and 200 $\mu$Ci) labeled sGC (12.5 and 25 $\mu$g) in the presence and absence of meta-PAL (100 $\mu$M) after separation in both subunits by SDS-PAGE.

![Figure 5](image-url)

**Figure 5**
Autoradiogram of $^3$H-meta-PAL labeled sGC after separation by SDS-PAGE. Lane 1: 12.5 $\mu$g sGC with $^3$H-meta-PAL without irradiation; Lane 2: 12.5 $\mu$g sGC with $^3$H-meta-PAL after irradiation; Lane 3: 2 $\mu$g in the presence of 100 $\mu$M unlabeled meta-PAL; Lane 4: 2 $\mu$g in the presence of 10 $\mu$M unlabeled meta-PAL; Lane 5: 2 $\mu$g in the presence of 100 $\mu$M ODQ; Lane 6: 2 $\mu$g in the presence of 100 $\mu$M YC-1.
51.8 kDa and three highly labeled bands in the Photo-Imaging-Plate of the TRIS-Tricine-gel in the molecular range between 6.2 and 14.7 kDa. These bands were identified and excised from the Coomassie-blue stained blot and sequencing of these bands showed, that they contain different fragments of both the α1- as the β1-subunit. Because the 3H-meta-PAL binds specifically to the α1-subunit of sGC and thereby all identified β1-sequences could be discarded, the overview of the determined α1-sequences shows, that the 3H-meta-PAL binds specifically to the CNBr VIII fragment, consisting of the amino acids 236–290 of the α1-subunit. To more closely identify the binding site of the 3H-meta-PAL, the single PTH-cycles from the sequencing of the CNBr VIII fragment were collected and the cysteines 238 and 243 of the α1-subunit were detected as binding sites of 3H-meta-PAL.

As shown in Fig. 9, these cysteines are conserved between the amino acid sequences of rat and human sGC [39,40] and this region could play an important role in regulation of sGC activity by this type of sGC. However, the putative target of bovine sGC differs from rat and human by the substitution of an arginine at position 238 instead of cysteine. As shown in previous studies with YC-1 [11] and verified in this study using crude bovine lung derived sGC extracts, we know that cysteine 238 is not essential for the stimulation of sGC by neither YC-1, nor BAY 41-2272, nor the PAL compound. However, is must be considered that the potencies of BAY 41-2272 on human, rat and bovine sGC could be different. For such comparison, the highly purified guanylate cyclases from these three species are needed. The mechanism of sGC stimulation is rather more complicated than simple interaction with one or two amino acids since the heme moiety bound to the His-105 of the β1-subunit is essential for activating the enzyme by YC-1, BAY 41-2272 and the PAL compound. There is a distance of 9 Å between the photolabile azido group and the pharmacophore of the compound. For this reason a covalent binding of the 3H-PAL to reactive cysteines slightly outside the binding pocket would not be surprising. Nevertheless, we assumed that Cys-238 and Cys-243 are in the direct region of access of 3H-PAL because no other labeled amino acids have been detected and a diffusion over a wide distance can be excluded.

Recent studies with YC-1 imply that YC-1 binds to an allostERIC site on sGC and thereby increases the maximal catalytic rate and sensitises the enzyme towards its gaseous activators NO and CO [11,13]. Studies about the kinetics and equilibria of sGC in the presence of YC-1 led to a mechanistic model, which attributes a crucial role to the proximal bond that connects the heme iron to the histidine 105 of the β1-subunit of sGC [41,42], but also requires protein control of the distal environment [43]. Firstly a small shift in the soret absorption feature of the sGC by YC-1 is observed. It is discussed, that this small
shift could be evoked by the replacement of the His-105 as the proximal heme ligand by another base, either another amino acid side chain or even YC-1 itself [43]. In contrast to [21] who postulated the YC-1 binding site in analogy to the forskolin binding site in the adenylate cyclase within the catalytic region of the α₁ subunit of the sGC in the region of cysteine 596, we showed that the new generated photoaffinity label for direct and NO-independent sGC stimulators binds to the cysteines 238 and 243 in the N-terminus of the α₁ subunit of the sGC and thereby in the near of the heme binding region. Hobbs [44] postulated an intramolecular sixth ligand of the heme, which oscillates on and off the sixth coordinate, thereby conferring some sort of ligand specificity (i.e. NO and CO). The binding of direct sGC stimulators to this site could block this intramolecular binding site and thereby simplify the binding of NO and CO to the enzyme. This model is concomitant with the sensitising of the enzyme towards NO by this class of stimulators. Very recently our findings have been supported [45] showing the important role of an additional heme binding domain for sGC activation by the NO-independent sGC stimulator YC-1. This domain is in the α-subunit in vicinity of the PAL labeled region.

**Conclusion**

In summary, using photoaffinity labelling, we identified the region of the cysteines 238 and 243 in the α₁ subunit of sGC as the target for NO-dependent sGC stimulators. However, the relevance of the identified region as a regulatory unit remains to be confirmed by mutational analysis and co-crystallization studies.

**Materials and Methods**

**Synthesis of YC-1 and the photoaffinity labels (PALs)**

YC-1 ((3-(5-hydroxymethyl-2-furyl)-1-benzylindazole) was synthesized as described [23] and was used as a 10 mM stock solution in DMSO. Three PAL candidates (BAY 50-6038, BAY 50-8364 and BAY 51-9491) were synthesized from the common intermediate BAY 41-2272 [24,25] (Fig. 1). The azide derivatives were obtained by deprotonation of the BAY 41-2272 primary amine with sodium hydride followed by reaction with the corresponding azide benzyol chloride. The ortho- and para-azidobenzoyl chlorides were synthesized by reaction of the corresponding ortho- para-azidobenzoic acid with thionyl chloride. In order to obtain the meta-azido derivative, the benzoyl chloride was synthesized in situ by reaction of the corresponding azidobenzoic acid [26] with 1-chloro-N,N-2-trimethylpropenylamine [27] (Fig. 1). The synthesis of the corresponding [3H]-BAY 51-9491 was accomplished as depicted in Fig. 2. Cyclization of the amine with ethyl cyano(cyclopropyl)acetate [28] afforded the 6-amino-4-hydroxypyrimidine derivative, which was converted to the bromo precursor by reaction with phosphoroxide tribromide. Br/³H exchange followed by formation of the amide bond as detailed above yielded the [³H]-BAY 51-9491.

**Purification of soluble guanylate cyclase (sGC) and determination of sGC activity**

sGC was highly purified from a baculovirus / Sf9 expression system and enzyme activity was measured by formation of [³²P]-cGMP from [α-³²P]-GTP modified according to Gerzer [29] in the presence of Mg²⁺ as the divalent metal cation as described [11]. Incubations were performed in the presence and absence of 1 mM DTT. All measurements were performed in duplicate and were repeated three times unless otherwise indicated. The specific activity of sGC was calculated as nmol cGMP formed per mg protein.

![Figure 8](image_url)

**Figure 8**

Course of the counted radioactivity of the single PTH-cycles of the sequencing of the CNBr VIII band. Presented are the measured dpm (decompositions per min, counting time 2 h). The pointed line represents the potential regression of the unspecific bound activity, which is washed of the membrane during the first cycles (“wash out”).

![Figure 9](image_url)

**Figure 9**

Sequence alignment of 3 α₁ subunits (human, rat, bovine) and 2 α₂ subunits (human, rat). Invariant amino acids are shown in red on yellow background. Amino acids building the consensus are shown in black on blue background and amino acids similar to the consensus are shown in black on red background. The two labeled cysteines are marked with arrows.
Stimulation of the crude sGC preparation from bovine lung by BAY 41-2272 in the absence and presence of DEA/NO (10 µM). The specific activity of sGC preparation is expressed as x-fold stimulation vs. basal activity (basal activity : 152 nmol/mg/min). The data presented represent means ± SEM, from 4 independent experiments performed in duplicate.

Spectroscopic studies
UV/Vis spectra were recorded from 300 to 600 nm on a DU 640 spectrophotometer ( Beckman, Munich, Germany). NO was introduced via an aqueous solution of DEA/NO. A 100 mM stock solution of meta-PAL in DMSO was prepared and added in a final concentration of 10 µM, resulting in a final DMSO concentration of 0.1% (v/v), which does not alter the properties of the enzyme.

Evaluation of CH- and NH-insertion qualities of the meta-PAL
Meta-PAL ( BAY 51-9491) was dissolved in diethylamine, dipropylamine, piperidine, toluene, or ethylbenzole in a concentration of 1 mg/ml and degassed by three thaw-freeze-cycles. 1 ml of each solution was given in a well of a 24 well PET plate ( Wallac, Turku, Finland) and irradiated with a UV hand lamp at 254 or 365 nm in a distance of 3 cm for 2 h (254 nm: N8K UV 254 nm hand lamp, Bendal, Wiesloch, Germany; 365 nm: Universal UV lamp, CAMAG, Berlin, Germany). Solutions were evaporated in a Speed Vac ( Bachofen, Reutlingen, Germany) and qualitatively and quantitatively analysed by LC/MS (column: symmetry C18, 2.1 x 150 mm; eluent: A: acetonitrile, B: 0.6 g HCl (30%) in 1 l MilliQ water; gradient: time 0: 10% A and 90% B flow: 0.6 ml/min, time 4 min: 90% A and 10% B flow: 0.6 ml/min, time 9.5 min: 10% A and 90% B flow: 0.8 ml/min; at 50°C). Resulting products were considered as NH- or CH-insertion products if their molecular weights correspond to the calculated weight of the insertion product and were given as % peak area of total peak area. To evaluate the irradiation time required for N2 degradation, meta-PAL (1 mg/ml) was dissolved in PAL buffer (final 50 mM TEA/HCl, 0.1 mM EGTA, 1 mM cGMP, 3 mM MgCl2, 200 µM GTP, pH 7.4) in the absence and presence of 0.3 mg/ml glucose oxidase as model protein and irradiated as described at 254 nm. Every minute an aliquot was removed and fragmented compounds were separated from meta-PAL by thin layer chromatography (stationary phase: silicagel 60 layered glass plate 5 x 10 cm (Merck, Darmstadt, Germany); mobile phase: acetoacetate).

Labeling of purified sGC
12.5 or 25 µg sGC (purity about 91%) were dissolved in PAL buffer (final 50 mM TEA/HCl, 0.1 mM EGTA, 1 mM cGMP, 3 mM MgCl2, 200 µM GTP, pH 7.4) and incubated with 200, 20 or 2 µCi 3H-meta-PAL (specific activity: 7.9 Ci/mmol = 0.29 MBq/nmol) in the presence or absence of 100 µM meta-PAL in a volume of 80 or 100 µl in a 24 well PET plate (5 min, 37°C). Samples were irradiated at 254 nm (distance 3 cm, 20°C, 15 min) and the reaction was stopped by adding 20 or 25 µl SDS-containing stop solution (312.5 mM TRIS/HCl / 10% (w/v) SDS / 50% (v/v) glycerine / 250 mM DDT / 0.025% (w/v) bromphenolblue, pH 6.8) and heating (5 min at 80°C). In a further study 12.5 µg sGC were incubated with 20 µCi 3H-meta-PAL in the presence and absence of the different sGC modulators as indicated, irradiated and stopped as described. For controls 12.5 µg sGC were incubated with 20 µCi 3H-meta-PAL without irradiation and 12.5 µg glucose oxidase were irradiated as described in the presence of 20 µCi 3H-meta-PAL. Separation was performed on a 7.5% SDS-PAGE (PROTEAN II cell, Bio-Rad, München, Germany) using a modified Laemmli method [30]. After electrophoresis, proteins were fixed for 20 min in methanol/acetic acid/ MilliQ water (30/10/60), dried and exposed to BAS-TR 20/25 Imaging-Plates ( Ray Test, Straubenhardt, Germany) for 15 days as described [31]. After exposure, the imaging plates were scanned ( BAS 5000 Scanner, Ray Test, Straubenhardt, Germany). Evaluation was performed by visual classification of radiographic intensities, displayed by use of pseudo-colours, starting with blue for lowest detectable concentrations up to red for highest concentrations as shown in each figure by a gradation bar.
CNBr fragmentation of labeled sGC

200 μg sGC were labeled as described with 1600 μCi 3H-meta-PAL, and the reaction was stopped by the addition of final 10% TCA. The labeled protein was precipitated (4°C, 30 min), centrifuged (14,000 rpm, 30 min; Centrifuge 5415C, Eppendorf, Hamburg, Germany) and washed twice with ice cold ethanol:ether (1:1). The protein pellet was dissolved in formic acid (70%) and reacted with a few crystals of CNBr in the dark (24 h, 20°C) under oxygen-free nitrogen. After evaporation in a Speed Vac (Bachofer, Reutlingen, Germany) for three times after the addition of 1 ml MilliQ water, the pellet was dissolved in 100 μl sample buffer (62.5 mM TRIS/HCl / 2% (w/v) SDS / 10% (v/v) glycine / 50 mM DDT / 0.0025% (w/v) bromophenolblue, pH 6.8), heated (5 min, 80°C) and the protein fragments were separated on a 10–20% gradient SDS-PAGE or a 16.5% TRIS- Tricine-gel [32], respectively. The digested protein fragments were transferred to a PVDF membrane (Trans-Blot® Electrophoretic Transfer Cell, Bio-Rad, München, Germany) [33], stained with 0.025% Coomassie-blue-R / 40% (v/v) MeOH and dissolved in acetonitril and MeOH and transferred to Combust-Cones, filled with rac 2211, Pharmacia) lyophilised, dissolved in acetonitril and MeOH and transferred to Combust-Cones, filled with two Combust-Pads (Packard, Illinois, USA). The radioactivity of each fraction was determined after combustion (Oxidizer model 307, Oximate 80, Packard, Illinois, USA) and dissolution of resulting gases in 15 ml scintillator (Monophase Scintillator, Packard) with a counting time of 2 h in a liquid scintillation counter (LS-6500, Beckmann, München, Germany).

Acknowledgements

The authors wish to thank Mrs. Dr. C. Robyr for the chemical synthesis of YC-1, and Mr. Heinrichs, Mrs. Mailé, Mrs. Keim, Mrs. Crummenier and Mr. Schneider for their outstanding technical assistance.

References

1. Wedel BJ, Garbers DL: New insights on the functions of the guanylyl cyclase receptors. FEBS Letters 1997, 410:29-33
2. Zabel U, Hausler C, Weeger M, Schmidt HH: Homodimerization of soluble guanylyl cyclase subunits. Dimerization analysis using a glutathione S-transferase affinity tag. J Biol Chem 1999, 274:18149-18152
3. Koglin M, Vehse K, Budaeus L, Scholz H, Behrends S: Nitric oxide activates the beta 2 subunit of soluble guanylyl cyclase in the absence of a second subunit. J Biol Chem 2001, 276:30737-30743
4. Lincoln TM: Cyclic GMP and mechanisms of vasodilation. Pharmac Ther 1989, 41:479-502
5. Moncada S, Higgs EA: Molecular mechanisms and therapeutic strategies related to nitric oxide. FASEB J 1995, 9:1319-1330
6. Zhuo M, Hawkins RD: Long-term depression: a learning-related type of synaptic plasticity in the mammalian central nervous system. Rev Neurosci 1995, 6:259-277
7. Arnold WP, Mittal CK, Katsuki S, Murad F: Nitric oxide activates guanylate cyclase and increases guanosine 3’:5’:cyclic-monophosphate levels in various tissue preparations. Proc Natl Acad Sci USA 1977, 74:3203-3207
8. Ko FN, Wu CC, Kuo SC, Lee FY, Teng CM: YC-1, a novel activator of platelet guanylate cyclase. Blood 1994, 84:4226-4233
9. Mulisch A, Baurerssch J, Schaefer A, Stasch JP, Kast R, Busse R: Effect of YC-1, an NO-independent, superoxide-sensitive stimulator of soluble guanylyl cyclase, on smooth muscle responsiveness to nitrovasodilators. Br J Pharmacol 1997, 120:681-689
10. Friebe A, Schultz G, Koelsing D: Sensitizing soluble guanylyl cyclase to become a highly CO-sensitive enzyme. EMBO J 1996, 15:663-668
11. Hoenicka M, Becker EM, Apeler H, Sirichoke T, Schröder H, Gerzer R, Stasch JP: Purified soluble guanylyl cyclase expressed in a baculovirus/SF9 system: stimulation by YC-1, nitric oxide, and carbon monoxide. J Mol Med 1999, 77:14-23
12. Briese B, Schmidt KU, Ulrich V: Activation of soluble guanylate cyclase by carbon monoxide and inhibition by superoxide anion, Eu. J Biochem 1990, 192:683-688
13. Friebe A, Koelsing D: Mechanism of YC-1-induced activation of soluble guanylyl cyclase. Mol Pharmacol 1998, 53:123-127
14. Stone JR, Marletta MA: Synergistic activation of soluble guanylate cyclase by YC-1 and carbon monoxide: implications for the role of cleavage of the iron-histidine bond during activation by nitric oxide. Chem Biol 1998, 5:255-261
15. Wu CC, Ko FN, Kuo SC, Lee FY, Teng CM: YC-1 inhibited human platelet aggregation through NO-independent activation of soluble guanylate cyclase. Br J Pharmacol 1995, 116:1973-1978
16. Wu CC, Ko FN, Teng CM: Inhibition of platelet adherence to collagen by cGMP-elevating agents. Biochem Biophys Res Commun 1995, 221:412-416
17. Teng CM, Wu CC, Ko FN, Lee FY, Kuo SC: YC-1, a nitric oxide-independent activator of soluble guanylate cyclase, inhibits platelet-rich thrombosis in mice. Eur J Pharmacol 1997, 320:161-166
18. Becker EM, Schmidt P, Schramm M, Schröder H, Walter U, Hoenicka M, Gerzer R, Stasch JP: The vasodilator-stimulated phosphoprotein (VASP); target of YC-1 and nitric oxide effects in human and rat platelets. Cardiovasc Pharmacol 2000, 35:390-397
19. Ruetten H, Mulisch A, Schoenfanger K, Martorana PA: The NO-independent activator of the soluble guanylyl cyclase YC-1.
pharmacological profile and tolerance studies. *Naunyn-Schmiedebers Arch Pharmacol* 1998, 358:310 20. Becker EM, Waelder F, Kast R, Robyr C, Hoenicka M, Gerzer R, Schroeder H, Stasch JP: Generation and characterization of a stable soluble guanylate cyclase-overexpressing CHO cell line. *Nitric Oxide* 1999, 3:55-66 21. Friese A, Russwurm M, Mergia E, Koesling D: A point-mutated guanylyl cyclase with features of the YC-1-stimulated enzyme: implications for the YC-1 binding site? *Biochem* 1999, 38:15253-15257 22. Stasch JP, Becker EM, Alonso-Alia C, Apeler H, Dembowsky K, Feurer A, Fürstner C, Apeler H, Dembowsky K, Feurer A, Fürstner C, Alonso-Alia C, Benet-Buchholz J, Ducke B, Feurer A, Fürstner C, Alonso-Alia C, Berterod M, Schramm M: NO-independent regulatory site on soluble guanylate cyclase. *Nature* 2001, 410:212-215 23. Yoshina S, Kuo SC: Studies on heterocyclic compounds. *XXXVII. Synthesis of furo[3,2-c]pyrazole derivatives. (5). Synthesis of alpha-methyl-1-(p-substituted phenyl)-3-phenyl-furo[3,2-c]pyrazole-5-acetic acids.* *Yukagaku Zasshi* 1978, 98:204-208 24. Straub A, Feurer A, Alonso-Alia C, Stahl E, Stasch JP, Perzborn E, Pleiji U, Schroeder H, Stasch JP, Alonso-Alia C, Benet-Buchholz J, Feurer A, Fürstner C, Alonso-Alia C, Stahl E, Steinke W, Straub A, Schramm M: NO-independent stimulators of soluble guanylate cyclase. *Bioorg Med Chem Lett* 2001, 11:781-784 25. Carnazza E, Aumelas A, Barberis C, Guillon G, Seyer R: A new series of photoactivatable and iodinatable linear vasopressor antagonists. *J Med Chem* 1994, 37:1841-1849 26. Devos A, Remion J, Frisque-Hesbain A, Colens A, Ghosez L: *Synthesis of acyl halides under very mild conditions.* *J Chem Soc Chem Commun* 1979, 24:1180-1181 27. Li Q, Chu DT, Claiborne A, Cooper CS, Lee CM, Raye K, Berst KB, Donner P, Wang W, Hasvold L, et al: *Synthesis and structure-activity relationships of 2-pyridones: a novel series of potent DNA gyrase inhibitors as antibacterial agents.* *J Med Chem* 1996, 39:3070-3088 28. Gerzer R, Boehme E, Hofmann F, Schultz G: Soluble guanylate cyclase purified from bovine lung contains heme and copper. *FEBS Lett* 1981, 132:71-74 29. Laemmli UK: Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* 1970, 227:680-685 30. Ahr Hj, Steinke W: Imaging Plate: Vasodilation and first experience with quantitative studies in whole-body autoradiography during drug development. *Xenobiotica* 1994, 24:371-378 31. Schagger H, Aguila H, von Jagow G: *Coomassie blue-sodium dodecyl sulfate-polyacrylamide gel electrophoresis for direct visualization of polypeptides during electrophoresis.* *Anal Biochem* 1988, 173:201-205 32. Towbin H, Staehlin T, Gordon J: Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. *Proc Natl Acad Sci USA* 1979, 76:4350-4354 33. Thomas R, Pfeuffer T: *Photoaffinity labeling of GTP-binding proteins.* *Meth Enzymol* 1991, 195:280-286 34. Pandurangi RS, Karra SR, Kuntz RR, Volkert WA: Recent trends in the evaluation of photochemical insertion characteristics of heterobifunctional perfluoroaryl azide chelating agents: biochemical implications in nucleic medicine. *Photochem Photobiol* 1997, 65:208-221 35. Garthwaite J, Southam E, Boulton CL, Nielsen EB, Schmidt K, Mayer B: Potent and selective inhibition of nitric oxide-sensitive guanylyl cyclase by 1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one. *Mol Pharmacol* 1995, 48:184-188 36. Brunner F, Schmidt K, Nielsen EB, Mayer B: Novel guanylyl cyclase inhibitor potently inhibits cyclic GMP accumulation in endothelial cells and relaxation of bovine pulmonary artery. *J Pharmacol Exp Ther* 1996, 277:48-53 37. Schrammel A, Behrends S, Schmidt K, Koelsch D, Mayer B: Characterization of 1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one as a heme-site inhibitor of nitric oxide-sensitive guanylyl cyclase. *Mol Pharmacol* 1996, 50:1-5 38. Nakane M, Iwai K, Saito S, Kuno T, Buehler W, Murad F: Molecular cloning and expression of cDNAs coding for soluble guanylate cyclase from rat lung. *J Biol Chem* 1990, 265:16841-16845 39. Zabel U, Weeger M, La M, Schmidt HHHW: Human soluble guanylate cyclase: functional expression and revised isoenzyme family. *Biochem* 1998, 335:51-57 40. Zhao Y, Marletta MA: Localization of the heme binding region in soluble guanylate cyclase. *Biochem* 1997, 36:15959-15964 41. Zhao Y, Schelvis JPM, Babcock GT, Marletta MA: Identification of histidine 105 in the beta1 subunit of soluble guanylate cyclase as the heme proximal ligand. *Biochem* 1998, 37:4502-4509 42. Kharitonov VG, Sharma VS, Magde D, Koelsing D: *Kinetics and equilibria of soluble guanylate cyclase ligation by CO: effect of YC-1.* *Biochem* 1999, 38:10699-10706 43. Hobbs AJ: *Soluble guanylate cyclase: the forgotten sibling.* 1997, 18:484-491 44. Koglin M, Behrends S, Scholz H: Cloning and expression of the rat D2 subunit of nitric oxide sensitive guanylyl cyclase and analysis of deletion mutants. *Naunyn-Schmiedebergs Arch Pharmacol* 2001, 363 (Supplement):187 45. Martin E, Lee Y-C, Murad F: YC-1 activation of human soluble guanylyl cyclase has both heme-dependent and heme-independent components. *Proc Natl Acad Sci USA* 2001, 23:12938