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Biochemistry and Pharmacology of Novel Anthranilic Acid Derivatives Activating Heme-Oxidized Soluble Guanylyl Cyclase

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Running title: Activators of heme-oxidized soluble guanylyl cyclase

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Non-standard abbrevations:

DMEM, Dulbecco's modified Eagle's medium; PBS, phosphate-buffered saline; RASMC, rat aortic smooth muscle cells; sGC, soluble guanylyl cyclase; HMR1766, 5-chloro-2-(5-chloro-thiophene-2-sulfonylamino-N-(4-(morpholine-4-sulfonyl)-phenyl)-benzamide sodium salt; S3448, 2-(4-chloro-phenylsulfonylamino)-4,5-dimethoxy-N-(4-(thiomorpholine-4-sulfonyl)-phenyl)-benzamide; IBMX, 3-isbutyl-1-methylxanthine; ODQ, 1H-[1,2,4]-oxdiazolo[3,4-a]quinoxalin-1-one; NS2028, 4H-8-bromo-1,2,4-oxadiazolo(3,4-d) benz(b)(1,4)oxazin-1-one; YC-1, 3-(5'-hydroxymethyl-2'furyl)-1-benzyl indazole; SNP, sodium nitroprusside; PIX, protoporphyrin IX; ZnP, zinc-protoporphyrin IX; DEANONOate, 2-(N,N-diethylamino)-diazenolate-2-oxide-Na^+-salt.

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ABSTRACT

The heme-enzyme soluble guanylyl cyclase (sGC) is an ubiquitous nitric oxide (NO) receptor, which mediates NO downstream signaling by generation of cyclic GMP. We studied the mechanism of action of the anthranilic acid derivatives HMR1766 (proposed international non-proprietary name: ataciguat sodium) and S3448 as a new class of sGC agonists. Both compounds activated different sGC preparations (purified from bovine lung, or crude from human corpus cavernosum) in a concentration-dependent and quickly reversible fashion (EC_{50} 0.5-10 µM), with mixed-type activation kinetics. Activation of sGC by these compounds was additive to activation by NO donors, but instead of being inhibited was potentiated by the heme-iron oxidants ODQ and NS2028, suggesting that the new compounds target the ferric heme sGC isoform. Protoporphyrin IX acted as a competitive activator and zinc-protoporphyrin IX inhibited activation of heme-oxidized sGC by HMR1766 and S3448, whereas heme-depletion of sGC by Tween 20-treatment reduced activation. Both compounds increased cGMP levels in cultured rat aortic smooth muscle cells, induced vasorelaxation of isolated endothelium-denuded rat aorta, porcine coronary arteries, and human corpus cavernosum (EC_{50} 1 to 10 µM), and elicited phosphorylation of the cGMP kinase substrate vasodilator-stimulated phosphoprotein (P-VASP) at serine239. HMR1766 i.v. bolus injection decreased arterial blood pressure in anaesthetized pigs. All these pharmacological responses to the new compounds were enhanced by ODQ and NS2028. Our findings suggest that HMR1766 and S3448 preferentially activate the NO-insensitive heme-oxidized form of sGC, which exists to a variable extent in vascular tissues, and is a pharmacological target for these new vasodilator drugs.

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INTRODUCTION

The heterodimeric heme-protein soluble guanylyl cyclases (E.C. 4.6.1.2. pyrophosphate lyase, cyclizing; sGC) functions as a receptor for the ubiquitous signaling molecule nitric oxide (NO). Binding of NO to the ferrous heme activates the enzyme for rapid catalysis of cyclic GMP (cGMP) formation from guanosine triphosphate (GTP) (Koesling and Friebe, 1999). The second messenger cGMP triggers several biological processes, such as a decrease in vascular tone and platelet activity, through interaction with cGMP-specific protein kinases, phosphodiesterases and ion channels (Munzel *et al.*, 2003). A unifying concept of the molecular requisites for sGC activation has been put forward (Ignarro *et al.*, 1984) (Ballou *et al.*, 2002). Activation by NO requires sGC heme-iron to be in the ferrous (II) state. Upon NO binding the iron is moved slightly out of the porphyrin plane, thereby releasing a distal histidine (His^{105} of the β_1-subunit) from iron coordination (Wedel *et al.*, 1994). This is considered to trigger subsequent intra-molecular re-arrangements influencing the catalytic center. Activation by NO is lost, but basal activity is preserved, if the heme is removed by mild detergents (Foerster *et al.*, 1996), or if the heme-iron is oxidized to the ferric state. Heme-iron oxidation is readily achieved by hexacyanoferrat(III) (Stone *et al.*, 1996) and by the newer type of sGC inhibitors, ODQ (Schrammel *et al.*, 1996) and NS 2028 (Olesen *et al.*, 1998). The apo-heme form of sGC can be activated by protoporphyrin IX and some other metal-free porphyrins (Wolin *et al.*, 1982), whereas heme (Fe(II)protoporphyrin) and Zn(II)porphyrin (Serfass and Burstyn, 1998), inhibit both, NO and protoporphyrin-sensitive sGC (Ignarro *et al.*, 1984). Interaction of sGC with activating and inhibiting porphyrins is believed to occur via heme-porphyrin exchange and binding of the porphyrins to the heme pocket (Ignarro *et al.*, 1984).

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In a screening approach for novel sGC activating compounds and the subsequent chemical optimization program we recently found anthranilic acid derivatives to potently activate isolated sGC (Schindler and Strobel, 2000)[1]. Surprisingly, activation of sGC by these compounds was increased by exposure to the sGC inhibitor ODQ, suggesting that they are able to activate ferric sGC. A biological significance of sGC redox-forms other than the ferrous heme sGC was not existing until recently, when the substance BAY58-2667 was described as the first NO and heme-independent activator of sGC which exhibited anti-platelet and vasodilator activity *in vitro* and *in vivo* (Stasch *et al.*, 2002). Activation of sGC by this compound was increased by removal of heme and by exposure to the sGC inhibitor ODQ (Schmidt *et al.*, 2004), thus indicating that ferric and/or apo sGC exists in intact tissues and can be pharmacologically targeted for activating cGMP dependent processes.

We here report on the effects of two anthranilic acid derivatives on bovine and human sGC redox forms and on their pharmacological activity in cultured smooth muscle cells, isolated blood vessels, and anaesthetized pigs. We provide experimental evidence that both compounds specifically activate ferric sGC[2].

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Materials and methods

Materials

Soluble GC was either purified from bovine lung (Mulsch *et al.*, 1989), or purchased from Alexis (Grünberg, Germany), which also provided ODQ and NS 2028. In some instances, partially purified enzyme was used. YC-1, HMR1766 and S3448 were synthesized at Aventis (Frankfurt, Germany). The structures are shown in figure 1. Stock solutions (10 mM) were prepared in DMSO, which did not exceed 2 % (v/v) in all experiments, and did not affect any of the parameters assessed. Due to its instability, DEA-NONOate (Alexis), dissolved in NaOH pH 9.0, was added immediately after sGC to assay mixtures. Firefly luciferase and NAD^+ were obtained from Roche (Mannheim, Germany). Sodium nitroprusside, protoporphyrin IX, zinc-protoporphyrin IX, and all other reagents were from Sigma (Dreieich, Germany). Human corpus cavernosum was obtained from gender transformation surgery. The identity of the patients was only known to the surgeons and was kept confidential.

Determination of sGC activity by chemiluminescence

sGC activity was measured by the conversion of GTP to pyrophosphate (PP_i) at 25°C for 60 min. PP_i formed was converted into ATP and nicotinamide mononucleotide in the presence of nicotinamide-mononucleotide adenylyl transferase (NAT) and nicotinamide adenosine dinucleotide (NAD^+). Reaction mixtures (volume 100 µl) contained 50 mM TEA, pH 7.6, 1 mM IBMX, 3 mM $MgCl_2$, 3 mM GSH, 0.1 mM GTP, 200 µM NAD^+ and 0.4 mU NAT. The reaction was started by the addition of sGC and was stopped by adding 50 mM EDTA. ATP was then determined by the firefly luciferase method (modified from (Barshop *et al.*, 1991)). The microtiter plates were placed in a luminometer (Lumistar Fa. BMG, Freiburg, Germany), and a volume of 20

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µl 100 mM $MgCl_2$ followed by 50 µl luciferase reaction mixture (62.5 mM Tris-acetate, pH 7.5, 1.9 mM EDTA, 50 µM DTT, 0.1% BSA, 150 µM Mg-acetate, 35 µM D(-)-luciferin (Photinus pyralis), 10 kU/ml luciferase, EC 1.13.12.7) was automatically injected. Raw data were obtained as relative light units (RLU) and were transformed into PP_i formation by means of a PP_i calibration curve. The specific enzyme activity was expressed as nmol PP_i produced per mg enzyme protein and minute (nmol mg^{-1} min^{-1}). All measurements were performed in duplicates and repeated up to 6 fold. Concentration response curves for determination of EC_{50} and V_{max} of activators were analyzed using the equation: $V=V_{max}*S/(K_M+ S)+BA$. Enzyme activation kinetics were assessed according to the Lineweaver-Burk transformation.

sGC activity assessed by formation of [^{32}P]cGMP

Activation of sGC was also assessed by conversion of [α-^{32}P]GTP as described previously (Mulsch *et al.*, 1989). Specific enzyme activity was expressed as nmol cGMP formed per minute per mg protein, if not indicated otherwise. Reversibility of sGC activation was tested with enzyme pre-exposed to NS2028 (10 min at 4°C) in order to achieve heme oxidation. Then 10 µM S3448 was added for further 5 min at RT. Thereafter the mixtures were diluted 10-fold into a solution for determination of sGC activity, with final S3448 concentration adjusted to either 1 or 10 µM, and enzymatic cGMP formation proceeded for 10 min at 37°C.

Removal of heme from sGC

Purified enzyme (15-17 µg aliquots) was incubated at 37° C in 30 mM Tris/HCl, 3 mM reduced glutathione, 3 mM $MgCl_2$ ("Tris/HCl buffer") and 2% (v/v) Tween 20 in a volume of 100 µl for 15 min (Foerster *et al.*, 1996). The detergent and heme were

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removed by quick spin Sephadex G-50 columns (Boehringer Mannheim) equilibrated with Tris/HCl buffer. Protein was eluted by centrifugation (4 min at 1100xg). Heme intact (native) sGC was subjected in parallel to the same experimental conditions, replacing Tween 20 by distilled water. The removal of heme was verified by UV-VIS spectroscopy in a Kontron 940+ double-beam spectrophotometer. Purified sGC exhibited an absorbance maximum at 430 nm (soret band) and a shoulder at 395 nm. Tween treatment abolished the soret band.

Isometric tension recordings

Endothelium denuded rings (3 mm length) from descending thoracic aorta of male Wistar rats (250-300 g) (RA), or from pig coronary artery (PCA), or isolated strips from human corpus cavernosum (HCC; 1 cm length, 4x3 mm width) were mounted in a thermostated (37°C) organ bath (Hugo Sachs Elektronik, March-Hugstetten, Germany) for isometric tension recording, as described previously (Wohlfart *et al.*, 1999).The tissues were equilibrated for 60 min in carbogenated (95% O_2; 5% CO_2) Krebs-Henseleit solution, pH 7.4 (composition in mM: Na^+ 144.0; K^+ 5.9; Cl^- 126.9; Ca^{2+} 1.6; Mg^{2+} 1.2; $H_2PO_4^-$ 1.2; SO_4^{2-} 1.2; HCO_3^- 25.0; D-glucose 11.1), in the presence of indomethacin (1 µM) or L-NAME (300 µM). During this time the resting tension was gradually increased to 2 g. Following a 30 min washout period, RA were contracted with 1 µM phenylephrine (PE), PCA with 10 nM U46619 (thromboxane receptor agonist), and HCC with noradrenaline (NA; 3 - 7 µM) to induce 80 % of maximal contraction achieved by 80 mM KCl. In some experiments with RA, ODQ (10 µM) or zinc-protoporphyrin (3 µM) was added to the organ baths 30 min before addition of the contractile agent. The porphyrin experiments were performed under red light. After development of a stable tension the relaxation to cumulative

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concentrations of S3448 or HMR1766 were recorded. Relaxation was expressed in percent (%) reversal of the increase in tone produced by the contractile agonists. Graphpad prism nonlinear regression analysis was used to calculate EC_{50} values.

cGMP formation in intact cells

Rat aortic vascular smooth muscle cells (RASMC) were cultured exactly as described previously (Ruetten *et al.*, 1996). Confluent cells grown in 6-well plates were rinsed two times with Hepes tyrode buffer (HTB) and pre-incubated in 1 ml HTB containing 0.1 mM IBMX and 200 U SOD. Ten µl of HMR 1766 (1 to 10 µM), SNP (3 to 30 µM) or the solvent DMSO were added, in the presence or absence of 1 µM ODQ. After 15 min the supernatant was removed, the cells quick-frozen with liquid nitrogen and stored at –20° C. For assaying cGMP the cells were thawed and 400 µl assay buffer (test kit) was added to each well. After thoroughly mixing for 30 min, a 100 µl aliquot was acetylated and cGMP concentrations were quantified with a commercial enzyme immuno assay (EIA, Amersham).

P-VASP formation

De-endothelialized aortic rings (8 per aorta) from Wistar rats were kept individually in 2 ml MEM with Penicillin/Streptomycin at 37°C for 2 h. Then they were transferred into 2 ml warmed (37°C) Hepes-Tyrode containing 30 µM N^G-nitro-L-arginine (L-NAG). After 5 min 10 µM NS2028 or solvent (0.1 % DMSO) was added to half of the rings, and after further 10 min solvent (0.1 % DMSO), SNP (1 µM), or S3448 (3 and 30 µM) were added. After 10 min the rings were frozen and homogenized in liquid nitrogen. SDS-PAGE electrophoresis and electroblotting was performed as described

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(Mulsch *et al.*, 2001). Immunoblotting was performed with a mouse monoclonal antibody (16C2) specific for P-VASP at serine239, as described (Mulsch *et al.*, 2001).

Determination of protein content

Protein pellets were dissolved in 100 µl 2 M NaOH at 95°C (2 h) and diluted with water (1:4 v/v). Protein concentrations were determined in a 10 µl aliquot according to Lowry's method, using bovine serum albumin (BSA) as standard.

Hemodynamic studies in anesthetized pigs

Randomized male pigs (German landrace 25 – 35 kg BW, n=5 per group) were anesthetized with pentobarbital sodium (i.v.-bolus of 19–21 mg/kg, followed by a continuous infusion of 16 to 19 mg pentobarbital/kg/h i.v. to maintain anesthesia) and artificially ventilated with room air and oxygen. Systolic and diastolic blood pressures as well as heart rate were continuously monitored with tip catheters (Millar PC 350) inserted into the left femoral artery and into the left ventricle via the right carotid artery. The right saphenous vein was cannulated and the test compounds were given as a single bolus in 5ml polyethylene glycol 400 (PEG400). Four treatment groups were evaluated: Placebo (PEG400), HMR 1766 3mg/kg, ODQ 1mg/kg, and the combination HMR1766 3mg/kg plus ODQ 1m/kg, respectively. The hemodynamic parameters were monitored for at least 120 min.

Statistical Analysis. Results are expressed as mean±SEM, if not indicated otherwise. The EC_{50} value for each experiment was obtained by logit-transformation. One-way ANOVA was used for comparisons of vascular responses and sGC activity. $P<0.05$ was considered significant. The Bonferroni correction was applied for

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comparison of multiple means.

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RESULTS

Activation of sGC from bovine lung by S3448 and HMR1766

In a screening approach the chemical class of anthranilic acid derivatives was found to potently activate soluble guanylyl cyclase (sGC) isolated from bovine lung. Two compounds, S3448 and HMR1766 free acid (figure 1) that were obtained in an extensive chemical optimization program (data presented at the Gordon Conference on Medicinal Chemistry, New London, USA 2004) induced a concentration-dependent 25 fold stimulation, with an EC_{50} of 0.68 and 0.51 µM, respectively (table 1) as determined in a novel bioluminescence based assay system. The NO-donor diethylamine-NONOate (DEANO) was less potent (EC_{50} of 1.54 µM) and even less effective (13.5 fold maximal increase). Interestingly, stimulation of sGC by a sub-maximally activating concentration of DEANO (1 µM) was additive to activation by HMR1766 throughout the entire concentration-response relationship (figure 2A), indicating independent activation mechanisms by both compounds. A Lineweaver-Burke plot showed apparent K_M values of 72.7±2.8 and 46.1±0.1 µM under basal and HMR1766 acid stimulated conditions, respectively (figure 2B). V_{max} values were 5.1±0.9 nmol mg^{-1} min^{-1} under basal and 52.9±11.4 nmol mg^{-1} min^{-1} under stimulated conditions. The extrapolated linear graphs intersected above the x-axis, indicating mixed-type activation kinetics (change in V_{max} and K_M).

Effect of sGC heme-iron oxidation state on activation by S3448

In order to assess the influence of the oxidation state of the sGC heme on activation by S3448 the activity bovine lung enzyme was tested in the absence and presence of NS2028 (10 µM), a substance known to inhibit NO-sensitive sGC by oxidation of its heme-iron (Olesen *et al.*, 1998). Maximal activation of the enzyme by 30 µM S3448

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was significantly increased in the presence of NS2028 by 70 % (Figure 3A). This effect was not restricted to the purified bovine enzyme, but was even more pronounced with sGC present in protein extract from human corpus cavernosum. As shown in figure 3B, the about 10-fold maximal activation of this enzyme by 30 µM S3448 was increased by more than 6-fold in the presence of 100 µM NS2028. These findings suggest that the novel compounds specifically activate the heme-oxidized ferric sGC. The difference in maximal sGC activation by S3448, and the different extent of the NS2028 effect between different sGC preparations likely represents a difference in relative abundance of ferrous vs. ferric sGC.

Reversibility of sGC activation by S3448

The reversibility of S3448-induced activation of ferric sGC was tested by analyzing, whether or not the extent of activation of the enzyme pre-incubated with S34348 is sensitive to dilution, according to the law of mass action. Therefore, NS2028-treated enzyme from bovine lung (Figure 4A) or human corpus cavernosum (Figure 4B) was incubated with and without 10 µM S3448 for 5 min at RT. Thereafter, the mixtures were diluted 10-fold and sGC activity determined, with final S3448 concentration adjusted to either 1 or 10 µM. As illustrated in figure 4B, activation of sGC in human tissue was independent of the S3448 concentration at pre-incubation (black columns), and depended entirely on S3448 concentration during the activity assessment (Figure 4B, gray columns). This finding indicates a complete and rapid reversibility of sGC activation in human corpus cavernosum.

The purified enzyme from bovine lung behaved slightly different to the crude human enzyme. After dilution of S3448-pre-incubated bovine lung sGC a lower enzyme activity was detected as compared to enzyme pre-incubated with solvent (Figure 4A).

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This finding indicates that activation of the heme-oxidized purified bovine enzyme is also rapidly reversible, but that in addition, pre-incubation with S3448 decreases subsequent activation of the enzyme by 3448. However, when pre-incubation was performed in the absence of ODQ no decrease in subsequent activation was seen (data not shown).

In further dilution experiments with HMR1766-pre-incubated purified bovine lung enzyme a curvilinear decline of cGMP production was observed, in contrast to a linear decrease observed with only solvent-pre-incubated enzyme activity, which was assessed in the absence of any activating compounds (data not shown). A linear decrease in cGMP production by the sGC-activator complex would be indicative of an irreversible binding, whereas the observed curvilinear decrease clearly indicated a reversible binding according to the law of mass action.

Effect of heme-iron oxidation state on maximal activation of sGC by S3448, sodium nitroprusside and protoporphyrin IX

We next studied the influence of sGC heme-iron redox states on maximal sGC activities elicited by S3448 (30 µM), sodium nitroprusside (SNP; 100 µM) and protoporphyrin IX (PIX; 1 µM). Therefore, the enzyme activity was assessed in the absence and presence of 10 µM (purified bovine enzyme) or 100 µM (crude human enzyme) NS2028. As illustrated in figure 5A, S3448 and SNP were approximately equi-efficient activators of the purified bovine enzyme, stimulating basal sGC activity maximally by about 17 and 14-fold. In contrast, with the crude human enzyme nearly 8 fold higher activity was elicited by SNP as compared to S3448 (figure 5B). The porphyrin stimulated the basal activity of the bovine sGC by 6.6 fold (figure 5A), and the human enzyme to only 30 % the activity seen with S3448 (figure 5B). In the

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presence of NS2028, the relative contributions of the sGC activities elicited by either agonist changed dramatically. S3448- and PIX-supported bovine and human enzyme activities increased in parallel, while SNP-supported activity was nearly abolished. The increase in S3448- and PIX- inducible sGC activity completely compensated for the decrease in SNP-stimulated activity. This is illustrated by the last pair of columns in figure 5AB, where we calculated the sum of S3448+SNP+PIX-supported sGC activities. This sum was constant, independent of the actual sGC heme-iron redox state.

Effect of protoporphyrin IX on activation of sGC by HMR1766 and S3448

Protoporphyrin IX (PIX) is known as an activator of the heme-depleted sGC (Friebe and Koesling, 1998). According to our finding that oxidation of sGC with NS2028 did not only increase activation by S3448, but also by PIX (figure 5), it might be possible that HMR1766/S3448 also activate the heme-free enzyme, and/or that PIX and S3448 target the same heme-redox form of sGC. Therefore, we studied the influence of PIX on activation of sGC by HMR1766/S3448. PIX (5 to 100 nM) induced a 7.7 to 9.7-fold increase in basal activity of purified bovine lung sGC (figure 6A). The concentration response curve of HMR1766 free acid in the presence of these PIX concentrations was shifted to the right in a concentration-dependent manner (figure 6A), and decreased the maximal activation achieved by 10 µM HMR1766 free acid. A similar result was obtained with the purified enzyme from bovine lung, which was assessed in the presence of 10 µM NS2028 to achieve complete heme-oxidation. PIX (1 µM) stimulated the basal activity of this enzyme by 45 fold, and significantly inhibited the S3448-induced activation (138 fold increase in basal activity at 30 µM) by 45 % (figure 6B). In accordance with this finding, also the activation of the NS2028

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(100 µM)-exposed human enzyme from corpus cavernosum by S3448 was significantly inhibited by 1 µM PIX to 57 % of its maximal activity (figure 6C). These findings show that protoporphyrin IX acts as a competitive agonist to HMR1766/S3448 with respect to activation of sGC.

Effect of heme-depletion and zinc-protoporphyrin IX on activation of sGC by S3448 and HMR1766

We then investigated whether the removal of the prosthetic heme of sGC by Tween 20 (Foerster *et al.*, 1996) would have an influence on the stimulatory behavior of S3448, and the competitive activation by PIX. Interestingly, heme-depleted bovine sGC, which showed a 90±5% reduction of maximal activation by 100 µM SNP (data not shown) was even more activated by S3448 than the native heme-intact enzyme (figure 7A). This activation by S3448 was completely abolished in the presence of 1µM PIX, which stimulated basal activity of the heme-depleted enzyme by 60 fold (figure 7B).

Since these findings would suggest that S3448 also activates the heme-free enzyme, we tested the influence of an acute addition of Tween 20 (0.5%) to the ongoing sGC activity test, 20 min after start of the reaction, and let cGMP formation proceed for further 40 min. As shown in figure 7C, addition of Tween immediately stopped cGMP formation by S3448 (1µM) and DEANO (10µM) stimulated native bovine sGC.

Zinc protoporphyrin IX, which is known as an inhibitor of NO-sensitive and PIX-sensitive sGC (Serfass and Burstyn, 1998) (Ignarro *et al.*, 1984) inhibited HMR1766 (10 µM) and S3448 (10 µM) induced activation of the native bovine sGC in a concentration-dependent manner (IC_{50} 0.6 µM), reaching almost complete inhibition at 10 µM (figure 7D). Zinc protoporphyrin IX also inhibited NO-dependent sGC

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activation by SNP, albeit with 10-fold lower potency (figure 7D).

Effect of HMR1766 on cGMP formation in smooth muscle cells

In cultured rat aortic smooth muscle cells (RASMC) HMR1766 elicited a concentration-dependent increase in cGMP formation, which (at 10 µM) rose to about 107 fold the solvent value (Table 2). In the presence of 1 µM ODQ basal cGMP levels were decreased by half, and sodium nitroprusside (SNP)-induced cGMP formation, which was comparable to HMR1766-induced cGMP formation, was completely blocked. In contrast, ODQ significantly increased the efficacy of HMR1766 induced cGMP formation by 4 to 5 fold (table 2).

Activation of cGMP-dependent proteinkinase I by S3448

The phosphorylation of the ubiquitous substrate of cGMP-dependent proteinkinase I (cGK-I), vasodilator-stimulated phosphoprotein (VASP), at serine 239 is a reliable biochemical monitor of cGK-I activity in cells and tissues (Oelze *et al.*, 2000). As illustrated by the representative western blot (Figure 8), incubation of rat aortic tissue with SNP (1 µM) or S3448 (3 and 30 µM) elicited a concentration-dependent phosphorylation of VASP, as measured by a specific antibody for $P_{Ser-239}$-VASP (Oelze *et al.*, 2000). Two P-VASP-positive peptide bands were detected at about 45 and 48 kDa, likely representing mono- and di-phosphorylated protein (Oelze *et al.*, 2000). P-VASP formation in response to SNP was completely blocked by addition of NS2028 (10 µM), whereas P-VASP formation induced by S3448 was significantly enhanced. According to a densitometric evaluation of both immunoreactive bands (Figure 8) the ratio of SNP(1 µM)- versus S3448(30 µM)-induced VASP phosphorylation decreased from 2:1 to 1:5.

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Vasorelaxation effects of S3448 and HMR1766

S3448 elicited a concentration-dependent relaxation in preconstricted endothelium-denuded rings from rat aorta (RA), porcine coronary artery (PCA), and strips from human corpus cavernosum (HCC) (Figure 9A). S3448 was most potent in PCA (EC_{50} 1.2 µM), less so in RA (EC_{50} 5.9 µM), and least in HCC (10 µM). Complete relaxation was achieved by 30 µM S3448 in PCA and 100 µM in RA, whereas we could not achieve more than 80 % relaxation with HCC due to solubility/solvent problems with S3448 at concentration exceeding 100 µM. The S3448-elicited relaxation of RA was significantly inhibited by pretreatment of the vessels with 3 µM zinc-protoporphyrin IX (Figure 9A, triangles). One hour pre-incubation of aortic rings with S3448 (30 µM), followed by 30 min washout had only a slight effect on the subsequent concentrations-response curve for S3448-induced relaxation (maximal relaxation 88-100 %, figure 9B). This finding demonstrates absence of in vitro tachyphylaxis to S3448. In contrast, pre-incubation of rat aortic rings with ODQ (10 µM; 50 min) significantly shifted the concentration-relaxation curve for HMR1766 to the left (EC_{50} 0.4 µM vs. 1.2 µM; $p < 0.05$) (Figure 9C).

Effect of HMR1766 on blood pressure

In anesthetized pigs with baseline systolic (126±5 mm Hg) blood pressure intravenous bolus application of placebo or ODQ (1mg/kg) induced weak changes in systolic blood pressure (Figure 9D). An intravenous dose of 3 mg/kg HMR1766 caused a long-lasting decrease in systolic blood pressure (Figure 9D). The HMR1766-induced hypotension was potentiated by the simultaneous injection of 1 mg/kg ODQ and developed to a maximal decrease of 49 mmHg within 15 min after administration. The duration of ODQ potentiation lasted for 120 min. Qualitatively

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similar effects were observed on diastolic blood pressure (data not shown). Interestingly, the heart rate (base line 94±3 beats/min) was only increased (+ 18 beats/min) during the peak hypotensive response to the combination of HMR1766 and ODQ.

DISCUSSION

The soluble guanylyl cyclases are a class of heterodimeric heme-proteins, which function as an important signaling element of the so-called L-arginine-NO-cGMP pathway (Friebe and Koesling, 2003). Nanomolar concentrations of NO are sufficient to saturate the ferrous heme-iron (Bellamy *et al.*, 2002), thereby inducing a conformational change that clears the catalytic center of the enzyme from intramolecular inhibition by the bound heme (Martin *et al.*, 2003). This results in a more than 100 fold increase in cGMP formation which elicits a variety of biological responses, such as vasorelaxation and neurotransmission (Krumenacker *et al.*, 2004). This peculiar property of sGC has been exploited in the past by a certain class of anti-hypertensive and anti-anginal compounds, the so-called NO donors/nitrovasodilators (Feelisch, 1998). However, their therapeutical value has been challenged recently by the finding that their chronic use is associated with increased superoxide formation (Munzel *et al.*, 2000). Thus, use of NO donors can give rise to formation of the deleterious peroxynitrite ($ONOO^-$), as has been shown in nitrate tolerant states, for instance (Munzel *et al.*, 2000). In order to avoid this unwanted side effect of NO-donors/nitrovasodilators, we (and others) looked for alternative sGC activators.

In the present study we analyzed the mechanism of action and characterized the

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pharmacological activity of two lead compounds of a new class of activators of sGC, HMR1766 and S3448 (figure 1). Both compounds stimulated the activity of purified sGC from bovine lung as well as that of the crude human enzyme in corpus cavernosum homogenate, in a concentration-dependent and quickly reversible fashion. Furthermore, they elicited cGMP increases in cultured vascular smooth muscle cells, activated cGMP-dependent proteinkinase in rat aorta, as detected by P-VASP formation, inhibited contraction of rat, porcine and human vascular tissue, and decreased systolic and diastolic blood pressure in anaesthetized rats. Most remarkably, the efficacy of both compounds was largely increased in the presence of inhibitors of NO-sensitive sGC, ODQ (Schrammel *et al.*, 1996) and NS2028 (Olesen *et al.*, 1998). Since these inhibitors prevent NO-dependent activation of sGC by oxidation of its heme-iron (Zhao *et al.*, 2000), our observation suggested that the anthranilic acid derivatives would specifically activate the ferric heme redox-form of sGC. It furthermore suggested that heme-oxidized enzyme was present to a small amount in isolated enzyme preparations and crude tissue homogenates. Closer examination of UV-VIS spectra of some sGC preparations revealed a shoulder at 395 nm, indicative of a small proportion of heme-oxidized enzyme. The finding was unexpected, because this redox-form of sGC until recently was not known to exist in living cells and tissues, in the absence of ODQ/NS2028. However, we could lend further support to this hypothesis by the observation that the increase in S3448 supported sGC activity assessed in vitro roughly accounted for the decrease in NO/SNP-supported activity in the presence of NS2028 (figure 5). This observation was compatible with a redox-switch from ferrous NO-sensitive to ferric HMR1766/S3448-sensitive sGC. This observation is not without precedent, since recently Stasch and colleagues reported similar findings with their new sGC activator

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BAY58-2667. This compound activated purified recombinant sGC from rat, and the efficacy of this compound was also increased by ODQ, albeit to a much lower degree than observed with HMR1766/S3448 (Stasch *et al.*, 2002). Guided by structural homology of BAY58-2667 with protoporphyrin IX, by competition experiments and using purified heme-depleted sGC these authors concluded that BAY2667 would preferentially activate the heme-free form of the enzyme. Protoporphyrin IX is known to activate the heme-depleted form of sGC (Foerster *et al.*, 1996), though it may also activate heme-intact forms by replacement of the heme (Ignarro *et al.*, 1984). We also observed that protoporphyrin IX acted as a competitive agonist to HMR1766/S3448 in the presence of ODQ/NS2028 (figure 6), and that zinc-protoporphyrin IX was a more potent inhibitor of HMR1766/S3448-dependent than of NO-dependent sGC activity (figure 7D). Thus, the possibility exists that HMR1766/S3448 activate the heme-free enzyme.

However, evidence against this mechanism of action was provided by the finding that HMR1766-induced cGMP formation immediately ceased after an acute depletion of the heme by addition of Tween 20 to an ongoing sGC enzyme reaction (figure 7C). Furthermore, S3448 even at 100 µM was not able to alter the activity of heme-depleted sGC in the presence of maximally activating concentration of protoporphyrin IX (figure 7B), while this was still possible with NS2028-treated enzyme (figure 6BC). Our findings are best explained by the assumption that the ODQ/NS2028-treatment to a certain extent also leads to loss of heme from the ferric enzyme, which would account for the increase in protoporphyrin IX-dependent sGC activity in the presence of NS2028, as shown in figure 5AB. Furthermore, it is conceivable that the ferric heme will exchange more easily with protoporphyrin IX than the ferrous enzyme, which explains the competitive antagonism of protoporphyrin IX with regard to

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HMR1766/S3448-supported sGC activity. Indeed, exchange of the ferric heme with protoporphyrin IX and other porphyrins has been demonstrated (Ignarro *et al.*, 1984;Stone *et al.*, 1996).

With regard to the mechanism of action discussed above, an exciting finding of the present study was that the new sGC activators exhibited potent pharmacological activity in vascular cells and tissues, and even living animals. This observation provides first evidence that heme-oxidized ferric sGC exists in vivo and can be specifically targeted by HMR1766/S3448. According to the cGMP measurements in smooth muscle cells, HMR1766 was roughly as efficacious as SNP (table2). This would suggest that ferric and ferrous (NO-sensitive) sGC were present in cultured cells in equal amounts. However, in the presence of ODQ, SNP-dependent cGMP formation was nearly completely blocked, while HMR1766-induced cGMP formation increased by 5-fold. According to this observation, the ferric enzyme would account for only 20 % of total sGC activity present in cultured rat aortic smooth muscle cells. We have now ready explanation for this deviation between maximally SNP- and HMR1766-supported cGMP formation in cultured cells. It may be that ODQ targets a pool of sGC which is not sensitive to SNP. Anyhow, a similar estimate for the proportion of HMR1766/S3448-sensitive vs. SNP-sensitive cGMP formation was obtained in isolated vascular tissues (data not shown), and from the comparison of SNP- and S3448-supported sGC activities in freshly homogenized human corpus cavernosum (figure 2B). Nevertheless, this relatively small prevalence of the ferric enzyme was still sufficient to accomplish a long-lasting drop in blood pressure in healthy young pigs treated i.v. by 3 mg/kg HMR1766 (figure 9C). Just as observed with the isolated enzyme, the vasodilator potency of HMR1766 in pigs *in vivo* was increased by ODQ, and the relaxant response to S3448 *in vitro* was blocked by zinc-

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protoporphyrin IX, supporting the concept that the ferric enzyme accounted for vasorelaxation by both compounds. Similar anti-platelet effects of HMR1766 observed *in vitro* and *in vivo* will be published in a separate paper (Koglin et al., in preparation). Further studies will have to show whether the proportion of ferrous and ferric enzyme is shifted in cardiovascular disease states associated with oxidative stress, and how patients may benefit from the new sGC activators under these conditions. In a recent paper, Witte and collaborators showed that HMR1766 significantly activated sGC assayed *in vitro* in internal mammary artery species from patients with coronary artery disease (with and without diabetes) (Witte *et al.*, 2004). The new compounds for the first time offer the unique opportunity to assess the redox-state of the heme-iron of sGC in living cells, tissues, intact organisms, and even in patients, and therefore should find broad application in pre-clinical and clinical studies. A recent study showed that long-term oral treatment of rats with HMR1766 attenuates monocrotaline-induced pulmonary arterial hypertension (M. Klein and U. Schindler, unpublished observation).

In conclusion, we here report on a new class of sGC activators, which specifically target the ferric heme-iron redox form of the enzyme. This form exists in vascular cells and tissues in sufficient concentration to accomplish cGMP-dependent pharmacological activity, such as vasorelaxation and hypotension. The compounds open a new and unmet approach to analyze the heme redox-state of sGC and should particular be beneficial in cardiovascular disease states associated with oxidative stress.

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Footnotes

[1] Part of this work was presented at the 1st International Conference on cGMP, Leipzig, 2003 (www.biomedcentral.com/browse/abstracts/CGMP/1)

[2] Part of this work was published previously in form of a Thesis by A. Töpfer [ISBN 3-930657-43-0].

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Legends to figures

Figure 1: Structural formula of the anthranilic acid derivatives (**A**) HMR1766 sodium salt (5-chloro-2-(5-chloro-thiophene-2-sulfonylamino-N-(4-(morpholine-4-sulfonyl)-phenyl)-benzamide) and (**B**) S3448 (2-(4-chloro-phenylsulfonylamino)-4,5-dimethoxy-N-(4-(thiomorpholine-4-sulfonyl)-phenyl)-benzamide).

Figure 2: (**A**) The concentration response curve for HMR1766-induced stimulation of sGC activity (0.5 µg sGC partially purified from bovine lung, 60 min, 25°C, PPi-assay) was obtained in the absence (■) or presence of 1 µM DEA-NONOate (❑). Symbols represent mean values±SEM from n=6 (HMR1766), and the mean value of one determination performed in duplicate (HMR1766+DEANO). (**B**) Lineweaver-Burke analysis of HMR1766-dependent sGC activation kinetics showing a double-reciprocal diagram of basal (❑) and HMR 1766 free acid (■, 0.3 µM)-stimulated sGC activity (V_a). sGC activity was determined with 0.85 µg sGC and different concentrations of GTP (6-200 µM). **B** shows summarized data from 3 independent experiments.

Figure 3: Influence of NS2028 on S3448-dependent activation of (**A**) sGC purified from bovine lung (20-40 ng) and (**B**) crude sGC of human corpus cavernosum extract (10 µg). The enzymatic formation of [^{32}P]cGMP was started by addition of enzyme diluted in assay buffer solution (see methods) to a reaction mixture containing [α-^{32}P]GTP (200 µM), S3448 (0.3-100 µM), 2% DMSO (●), or (**A**) 10 µM, or (**B**) 100 µM NS2028 (○), and the reaction proceeded for 10 min at 37°C. [^{32}P]cGMP was then isolated, quantified by scintillation counting, and the specific sGC activity calculated (nmol cGMP formed per mg of protein per min incubation time). Mean values±SEM from 4 experiments performed in duplicate.

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Figure 4: Reversibility of S3448-induced activation of NS2028-treated sGC from (**A**) bovine lung and (**B**) human corpus cavernosum. The enzyme was incubated with (**A**) 10 µM, or (**B**) 100 µM NS2028 for 10 min at 4°C, then with (black columns) or without 10 µM S3448 (gray columns) for further 5 min at RT. Thereafter the mixtures were diluted 10-fold into a solution for determination of sGC activity, with final S3448 concentration adjusted to either 1 or 10 µM. Enzymatic cGMP formation proceeded for 10 min at 37°C, [^{32}P]cGMP was detected, and specific sGC activity was calculated. Mean values±SEM from n=4.

Figure 5: Influence of NS2028 on sGC activation by S3448 (30 µM), sodium nitroprusside (SNP; 100 µM), and protoporphyrin IX (PIX; 1 µM). The activity of sGC from (**A**) bovine lung, and (**B**) human corpus cavernosum was determined as described in fig. 3, in the absence (open columns), or presence (closed columns) of NS 2028. The last pair of columns represents the calculated sum of sGC activities (S3448 + SNP + PIX). Mean values±SEM from n=4.

Figure 6: Interference of protoporphyrin IX (PIX) with activation of sGC by (**A**) HMR1766 acid and (**B, C**) S3448. (**A**) The activity (PP_i formation from GTP) of partially purified bovine lung sGC (0.17 µg/ml) was assessed in the presence of different concentrations of PIX (5 – 100 nM) and HMR1766 acid, as indicated. Representative experiment. (**B**) The activation of NS2028(10 µM)-treated bovine lung sGC (25 ng) by S3448 (1-30 µM) was assessed in the absence (hatched columns) or presence (closed columns) of 1 µM PIX. Mean values±SE of n=2. (**C**) The activation of NS2028(100 µM)-treated human corpus cavernosum sGC (10 µg) by S3448 (1-30

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µM) was assessed in the absence (hatched columns) or presence (closed columns) of 1 µM PIX. Mean±SE of n=2.

Figure 7: Effect of heme-depletion and zinc-protoporphyrin IX on activation of sGC by S3448 and HMR1766

(**A**) Activation of native (open columns) and heme-depleted (0.5% tween 20-treated; closed columns) bovine lung sGC (0.4 ng) by S3448 and YC-1 (100 µM). Mean±SE of n=2.

(**B**) Activation of heme-depleted bovine lung sGC (50 ng) by S3448 was assessed in the presence of 1 µM protoporphyrin IX (PIX). Mean±SE of n=2.

(**C**) Tween 20 immediately blocks sGC activation by S3448 (□,■) and DEA-NONOate (○,●). Time course of PPi formation by partially purified bovine lung sGC during 60 min, with (○,□) or without (●,■) addition of 0.5% Tween 20 after 20 min. Mean±SE of n=3.

(**D**) Inhibition by zinc protoporphyrin of activation of bovine lung sGC by HMR1766 free acid (10 µM; ●), S3448 (10 µM; ○), and SNP (100 µM;▲).

Figure 8: S3448-elicited phosphorylation of VASP in rat aorta.

De-endothelialized rat aortic rings were incubated in the absence and presence of NS2028 (10 µM), and after 10 min were stimulated for 10 min with either solvent (0.1 % DMSO; "Con"), SNP, or S3448, as indicated. Tissues were homogenized and subjected to western-blot analysis for P-VASP. The representative immunoblot shows the two immunoreactive bands of P-VASP at ~ 45 kDa. The column diagram below represents a densitometric evaluation (mean ± SEM) of 3 different experiments performed with rings from 3 rats. * : $p < 0.05$ *vs*. control; §: $p < 0.05$ *vs*. -NS2028.

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Figure 9: Vasodilatory and hypotensive effects of S3448.

(**A**) Concentration-dependent relaxation by S3448 of PE(1 µM)-contracted endothelium-denuded rat aorta (RA) (●), U46619(1 µM)-contracted porcine coronary artery (O), and noradrenaline(3 - 7 µM)-contracted human corpus cavernosum (■). The relaxation of RA was completely inhibited by 3 µM zinc-protoporphyrin added 30 min before PE (▲). Data show mean±SE of 3-4 different vessels from either species.

(**B**) Effect of 60 min in vitro pre-treatment with either solvent (□), or 30 µM S3448 (■) on relaxation of PE (1 µM) constricted rat aorta by S3448. Mean±SE of up to 8 rings from 3 rats.

(**C**) Effect of 50 min in vitro pre-treatment with either solvent (O), or 30 µM ODQ (●) on relaxation of PE (0.1 µM) constricted rat aorta by HMR1766. Mean±SE of 6 rings from 2 rats.

(**D**) Effect of HMR1766 on systolic blood pressure in pentobarbital anaesthetized mini-pigs. HMR 1766 (●) was given at a dose of 3 mg/kg i.v. or in the combination (■) of HMR1766, 3 mg/kg i.v. plus ODQ, 1 mg/kg i.v. respectively, each in 5 pigs. PEG-placebo (O) and ODQ (□) treated mini-pigs served as control groups. Data are given as difference to the placebo (126±2), ODQ (126±5), HMR1766 (116±6 mm Hg) and HMR1766/ODQ (131±6 mm Hg) baseline pressures.

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TABLES

Compound	EC_{50} (μM)	V_{max} fold basal
S3448	0.68±0.17 (5)	24.5±1.6 (5)
HMR1766 acid	0.51±0.06 (7)	25.6±0.8 (7)
DEANO	1.54±0.02 (3)	13.5±1.0 (3)

Table 1: Effect of anthranilic acid derivatives and DEANONOate (DEANO) on enzymatic activity of partially purified sGC from bovine lung, determined by pyrophosphate (PPi) formation from GTP by 0.17 μg/ml sGC. Maximal enzyme activity (V_{max}) is expressed as fold stimulation of basal activity. Mean values ± SEM of n experiments, as indicated in parentheses.

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Compound	Concentration	cGMP	
	(µM)	**-** ODQ (pmol/mg protein)	+ ODQ (% of **-** ODQ)
Solvent		0.80±0.08	53 %
HMR 1766	1	14.70±1.00	445 %
HMR 1766	3	46.50±2.32	451 %
HMR 1766	10	85.70±2.78	485 %
SNP	3	45.50±4.20	1.4 %
SNP	10	43.00±3.57	3.2 %
SNP	30	70.10±3.61	2.6 %

Table 2: Effect of HMR1766 and SNP on cGMP concentrations (pmol mg^{-1} protein; mean ± SE, n=3) in SMC from rat aorta in the absence (**-** ODQ) and presence (+ ODQ) of 1 µM ODQ. The cells were exposed to sGC activators for 10 min in the presence of 0.1 mM IBMX.

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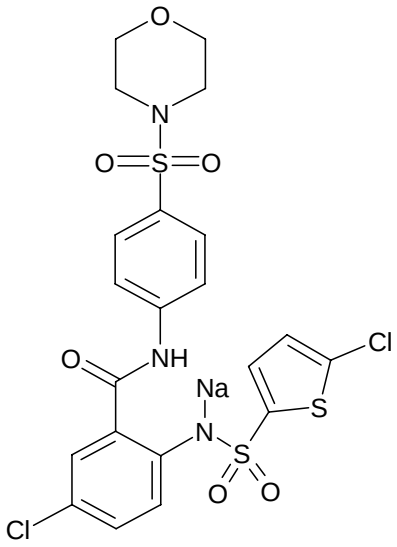
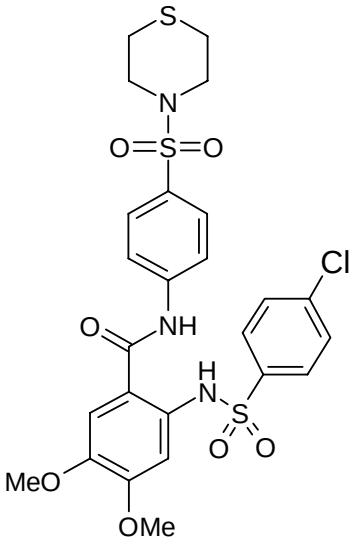
Fig. 1



HMR1766



S3448

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Fig. 2

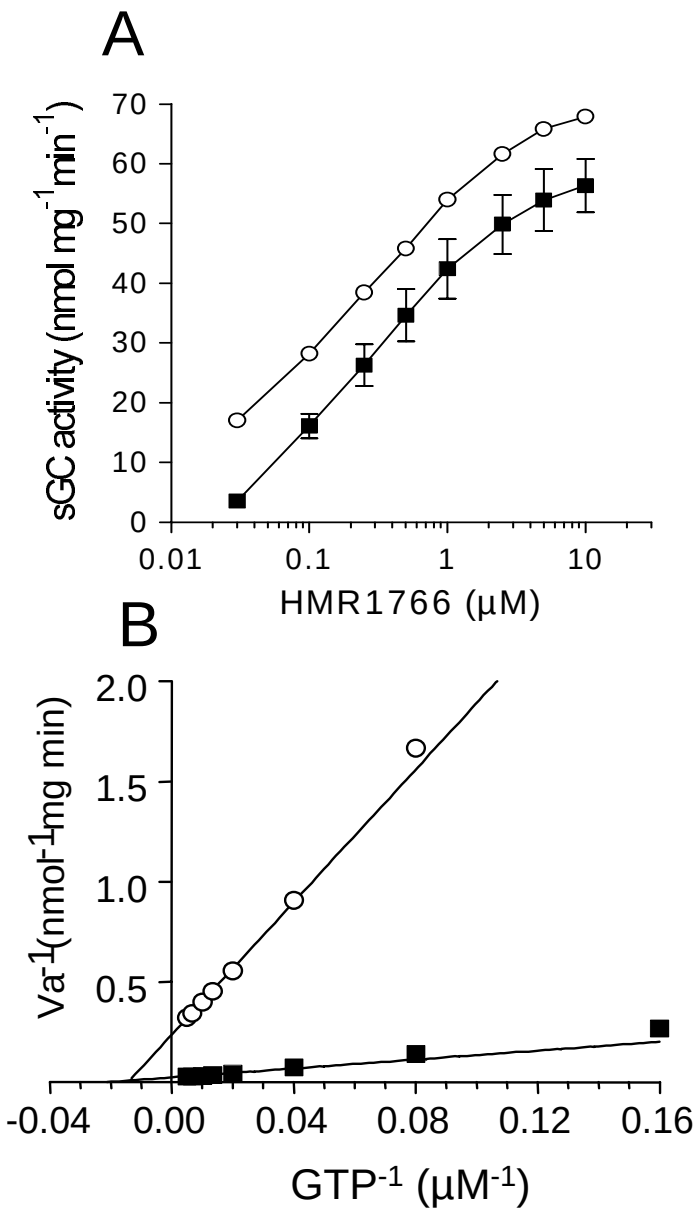

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Fig. 3

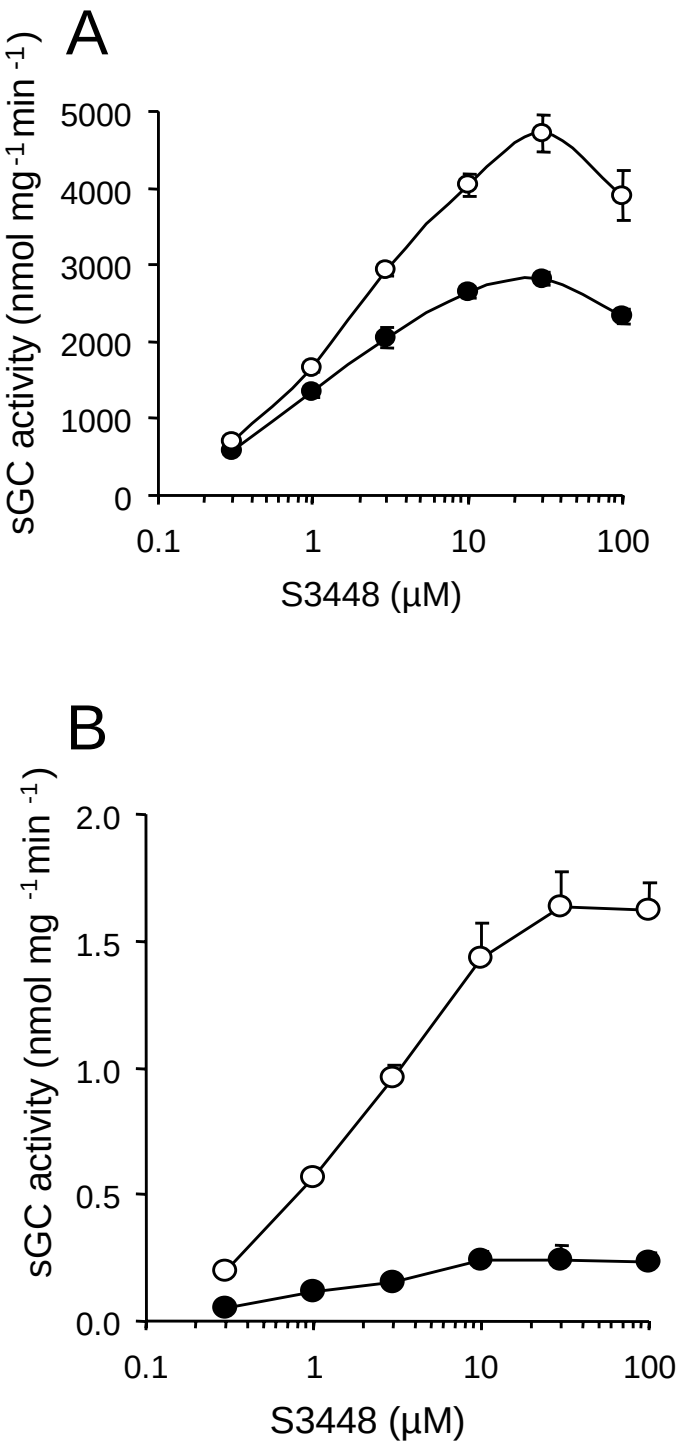

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Fig. 4

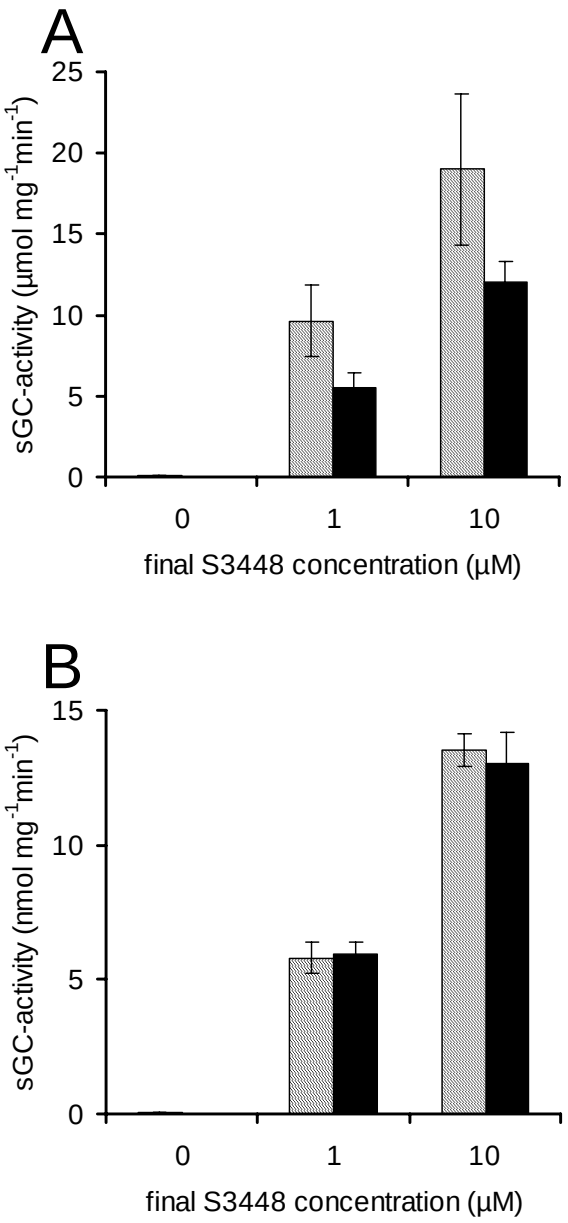

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Fig. 5

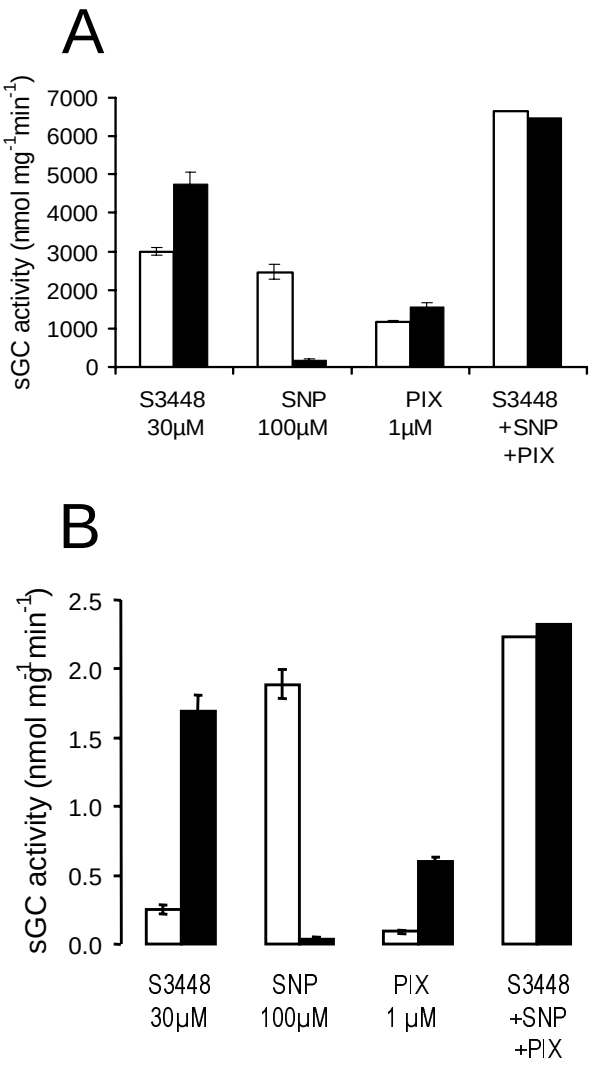

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Fig. 6

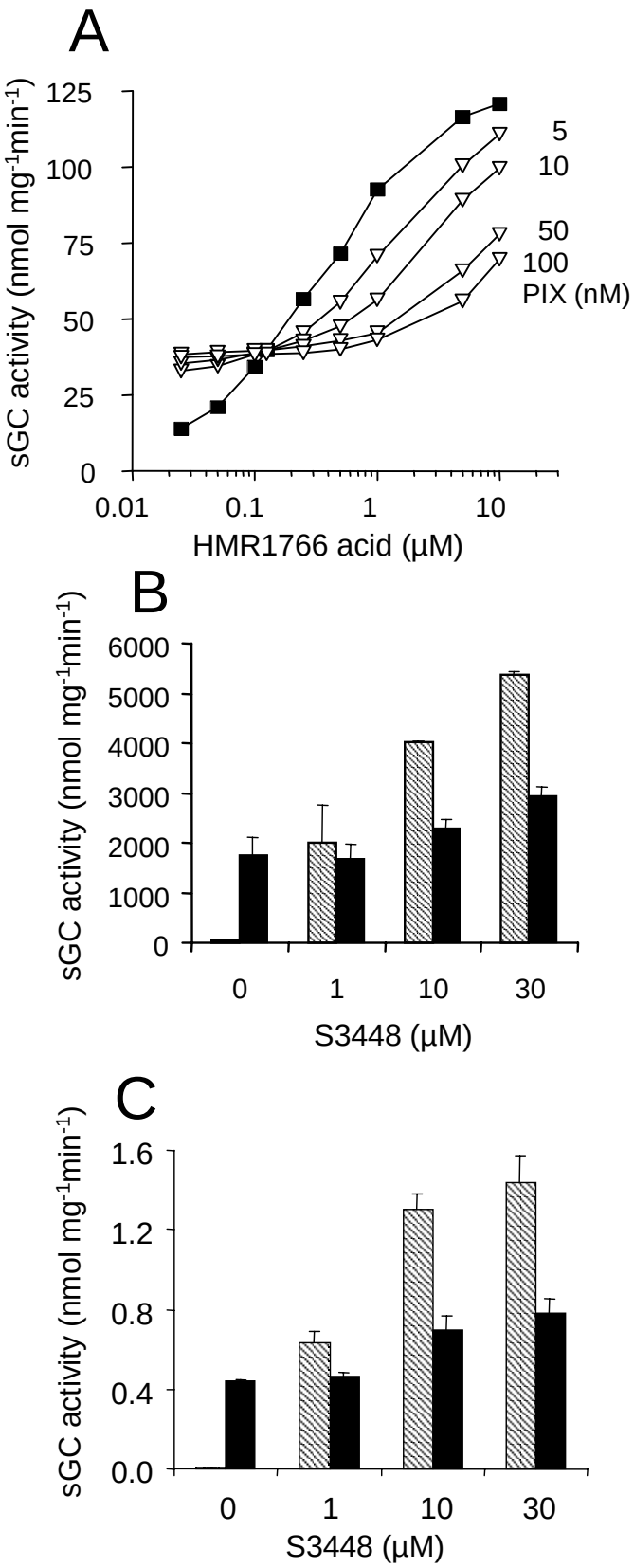

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Fig. 7

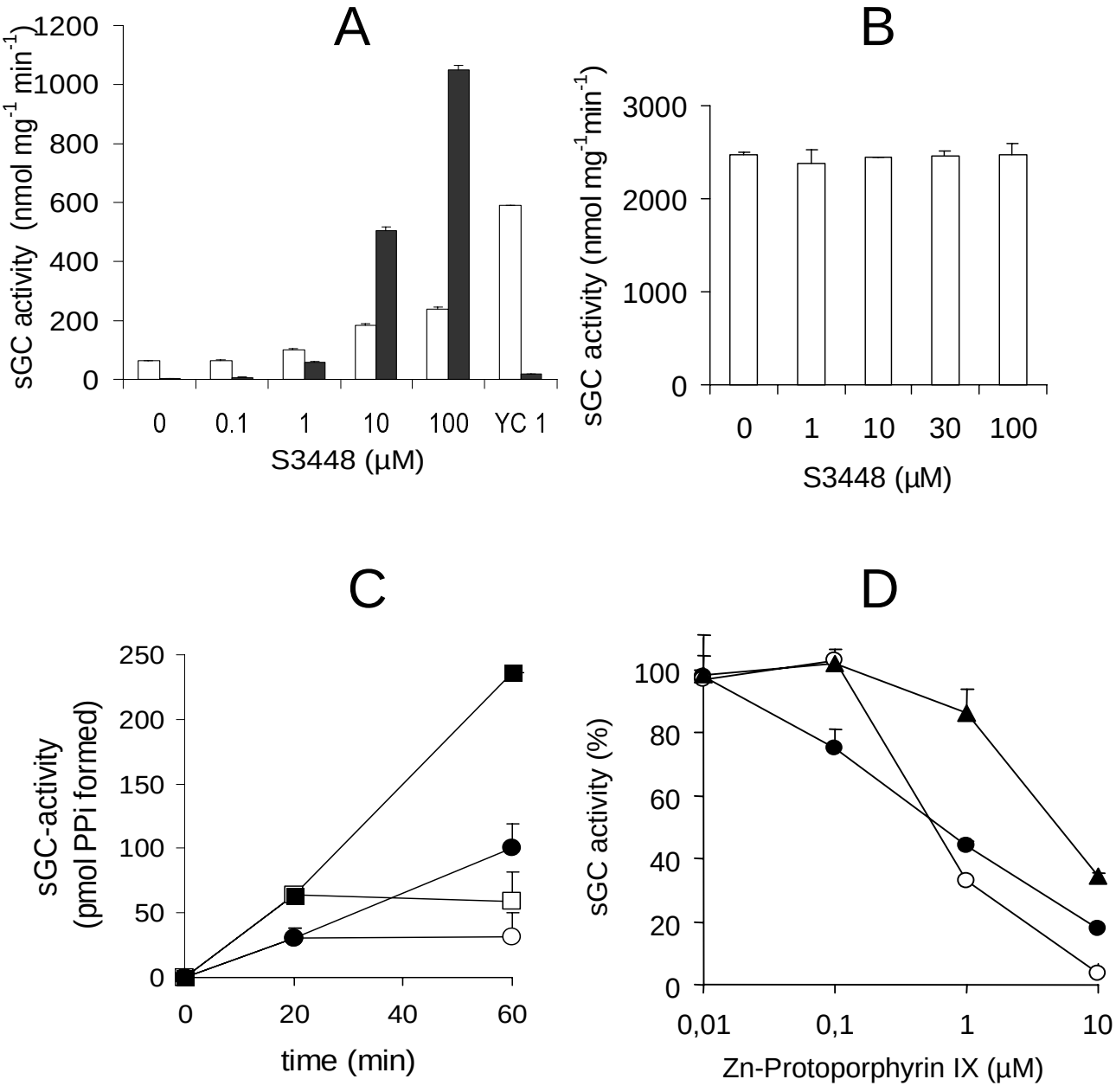

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Fig. 8

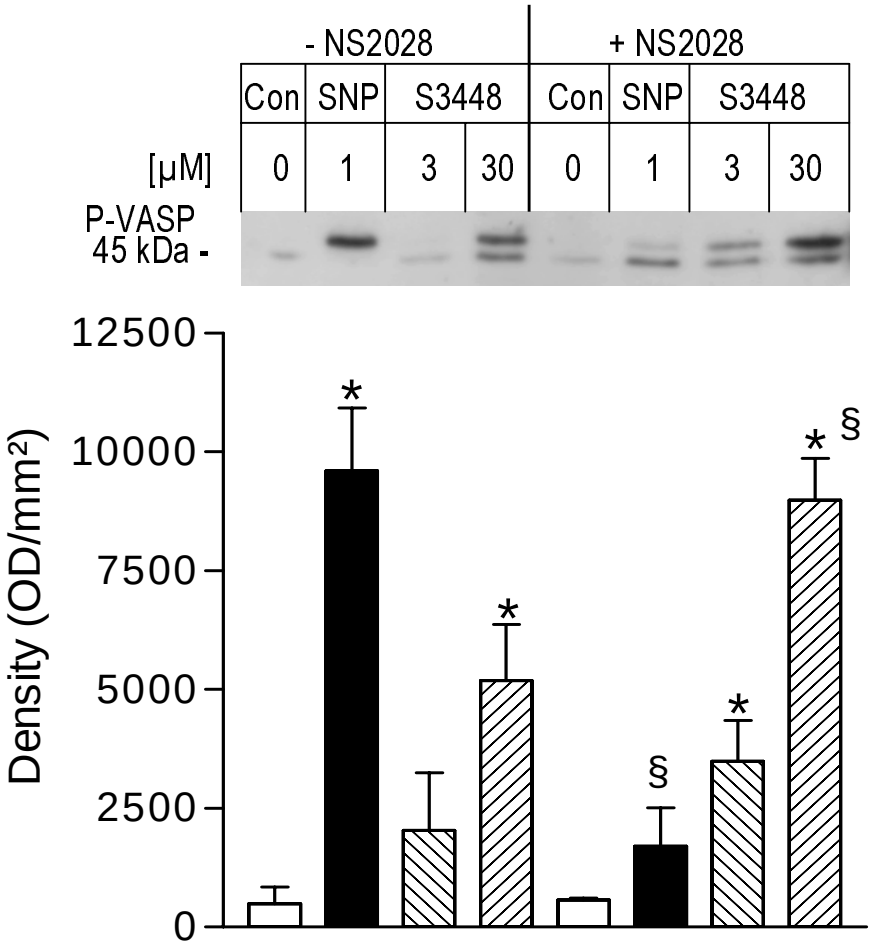

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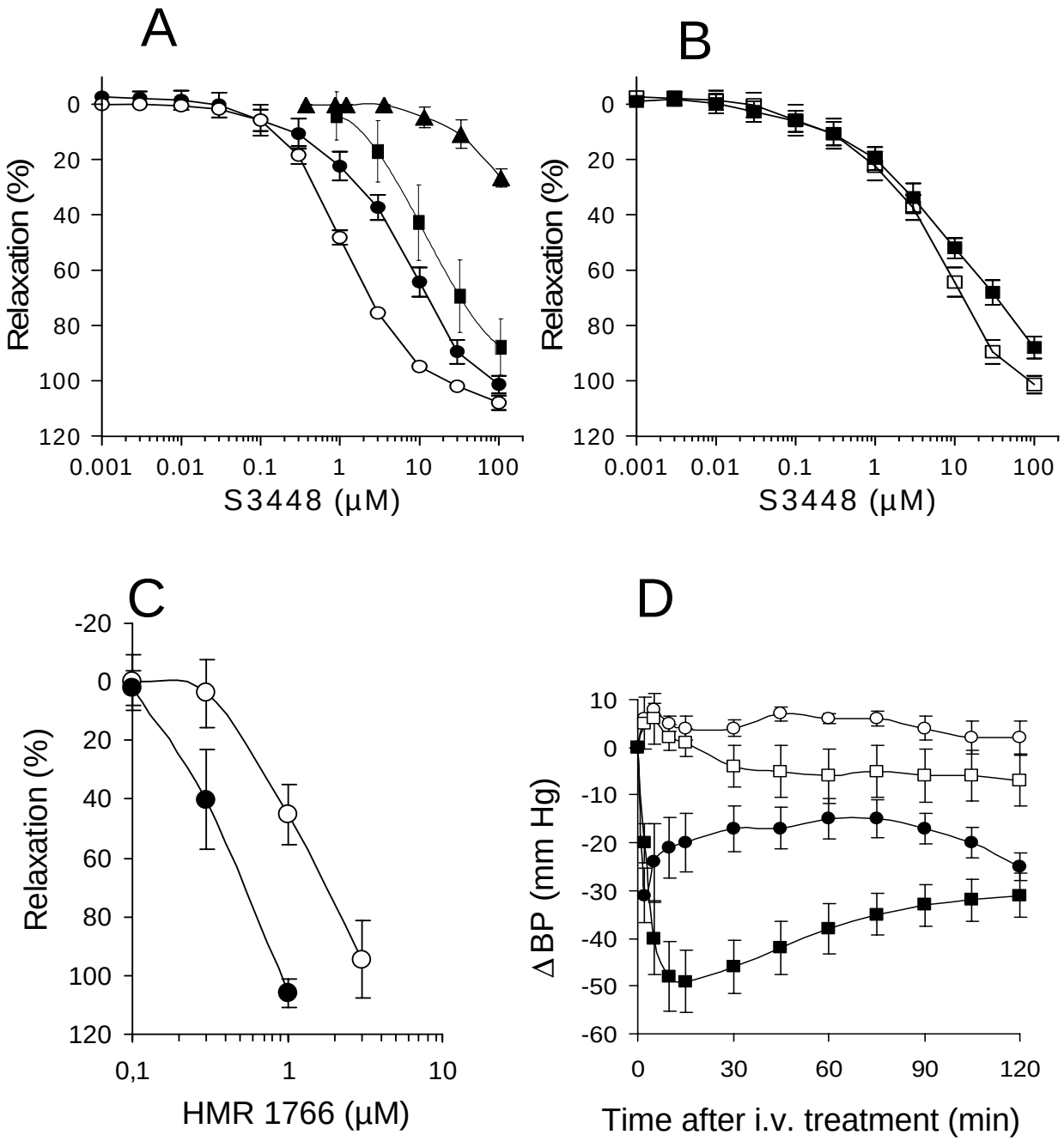
Fig. 9



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