

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

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ABSTRACT

The heme-enzyme soluble guanylyl cyclase (sGC) is an ubiquitous NO receptor, which mediates NO downstream signaling by the generation of cGMP. We studied the mechanism of action of the anthranilic acid derivatives 5-chloro-2-(5-chloro-thiophene-2-sulfonylamino)-*N*-(4-(morpholine-4-sulfonyl)-phenyl)-benzamide sodium salt (HMR1766) (proposed international nonproprietary name, ataciguat sodium) and 2-(4-chloro-phenylsulfonylamino)-4,5-dimethoxy-*N*-(4-(thiomorpholine-4-sulfonyl)-phenyl)-benzamide (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 ($EC_{50} = 0.5\text{--}10\text{ }\mu\text{M}$), with mixed-type activation kinetics. Activation of sGC by these compounds was additive to activation by NO donors, but instead of being inhibited, it was potentiated by the heme-iron oxidants 1*H*-[1,2,4]-oxdiazolo[3,4-*a*]quinoxalin-1-one (ODQ) and 4*H*-8-bromo-1,2,4-oxadiazolo(3,4-*d*) benz(b)(1,4)oxazin-1-one

(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 at Ser239. HMR1766 intravenous bolus injection decreased arterial blood pressure in anesthetized pigs. All of 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.

The heterodimeric heme-protein soluble guanylyl cyclases (E.C. 4.6.1.2. pyrophosphate lyase, cyclizing; sGC) function

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ABBREVIATIONS: sGC, soluble guanylyl cyclase; DMSO, dimethyl sulfoxide; RASMC, rat aortic smooth muscle cell; 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-isobutyl-1-methylxanthine; ODQ, 1*H*-[1,2,4]-oxdiazolo[3,4-*a*]quinoxalin-1-one; NS2028, 4*H*-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; DEA-NONOate, 2-(*N,N*-diethylamino)-diazolate-2-oxide- Na^+ salt; RT, room temperature; P-VASP, phosphorylated-vasodilator-stimulated phosphoprotein; PP_i, pyrophosphate; RA, rat aorta; PCA, pig coronary artery; HCC, human corpus cavernosum; PE, phenylephrine; DEANO, diethylamine-NONOate; U46619, 9*a*,11*a*-methanoepoxy-15-hydroxyprosta-5,13-dienoic acid; BAY 58-2667, 4-[[[4-carboxybutyl][2-[(4-phenethyl-benzyl)oxy]phenethyl]amino)methyl] benzoic acid.

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 (His105 of the β_1 subunit) from iron coordination (Wedel et al., 1994). This is considered to trigger subsequent intramolecular rearrangements 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 hexacyanoferrate(III) (Stone et al., 1996) and by the newer type of sGC inhibitors, ODQ (Schrammel et al., 1996) and NS2028 (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).

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 et al., 2000). 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 did not exist until recently, when the substance BAY 58-2667 was described as the first NO- and heme-independent activator of sGC that exhibited antiplatelet and vasodilator activity in vitro and in vivo (Stasch et al., 2002). Activation of sGC by this compound was increased by the 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 report here 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 anesthetized pigs. We provide experimental evidence that both compounds specifically activate ferric sGC.

Materials and Methods

Materials. sGC was either purified from bovine lung (Mulsch et al., 1989) or purchased from Alexis (Grünberg, Germany), which also provided ODQ and NS2028. In some instances, partially purified enzyme was used. YC-1, HMR1766, and S3448 were synthesized at Aventis (Frankfurt, Germany). The structures are shown in Fig. 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. Because of its instability, DEA-ONONOate (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 and NAD^+ . Reaction mixtures (volume, 100 μl) contained 50 mM tetraethylammonium, pH 7.6, 1 mM IBMX, 3 mM MgCl_2 , 3 mM glutathione, 0.1 mM GTP, 200 μM NAD^+ , and 0.4 mU nicotinamide-mononucleotide adenylyl transferase. The reaction was started by adding 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 μl of 100 mM MgCl_2 followed by 50 μl of luciferase reaction mixture [62.5 mM Tris-acetate, pH 7.5, 1.9 mM EDTA, 50 μM dithiothreitol, 0.1% bovine serum albumin, 150 μM magnesium acetate, 35 μM D(-)-luciferin (*Photinus pyralis*), and 10 kU/ml luciferase, EC 1.13.12.7] was automatically injected. Raw data were obtained as relative light units and were transformed into PP_i formation by means of a PP_i calibration curve. The specific enzyme activity was expressed as nanomoles of PP_i produced per milligram of enzyme protein per minute. All measurements were performed in duplicate and were repeated up to 6-fold. Concentration-response curves for the determination of EC_{50} and V_{max} values of activators were analyzed using the equation $V = V_{\text{max}} \times S / (K_M + S) + BA$, where S is concentration of substrate and BA is basal enzyme activity. Enzyme activation kinetics were assessed according to the Lineweaver-Burk transformation.

sGC Activity Assessed by the Formation of [^{32}P]cGMP. Activation of sGC was also assessed by the conversion of [$\alpha\text{-}^{32}\text{P}$]GTP, as described previously (Mulsch et al., 1989). Specific enzyme activity was expressed as nanomoles of cGMP formed per minute per milligram of protein, if not indicated otherwise. Reversibility of sGC activation was tested with enzyme pre-exposed to NS2028 (10 min at 4°C) 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 removed by quick-spin Sephadex G-50 columns (Boehringer Mannheim, Mannheim, Germany) equilibrated with Tris-HCl buffer. Protein was eluted by centrifugation (4 min at 1100g). Heme-intact (native) sGC was subjected in parallel to the same experimental conditions, replacing Tween 20 with distilled water. The removal of heme was verified by UV-visible spectroscopy in a Kontron 940+ double-beam spectrophotometer (Kontron Instruments, Watford, Herts, UK). Purified sGC exhibited an absorbance maximum at 430 nm (soret band) and a shoulder at 395 nm. Tween treatment abolished the soret band.

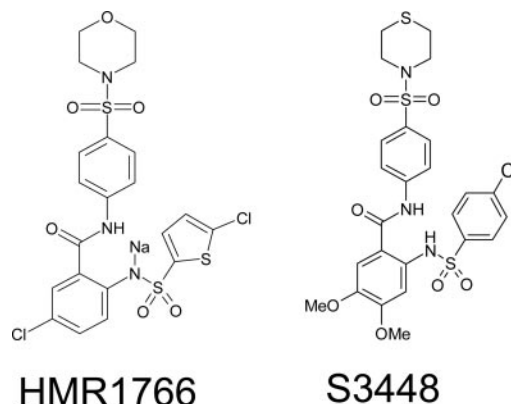


Fig. 1. Structural formula of the anthranilic acid derivatives HMR1766 sodium salt (A) and S3448 (B).

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, 4 × 3 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% O₂/5% CO₂) Krebs-Henseleit solution, pH 7.4 (composition: 144.0 mM Na⁺, 5.9 mM K⁺, 126.9 mM Cl[−], 1.6 mM Ca²⁺, 1.2 mM Mg²⁺, 1.2 mM H₂PO₄[−], 1.2 mM SO₄^{2−}, 25.0 mM HCO₃[−], and 11.1 mM D-glucose), in the presence of indomethacin (1 μM) or N^ω-nitro-L-arginine methyl ester (300 μM). During this time, the resting tension was gradually increased to 2 g. After a 30-min washout period, RAs were contracted with 1 μM phenylephrine (PE), PCA with 10 nM U46619 (thromboxane receptor agonist), and HCC with noradrenaline (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 the addition of the contractile agent. The porphyrin experiments were performed under red light. After the development of a stable tension, the relaxation to cumulative concentrations of S3448 or HMR1766 was recorded. Relaxation was expressed as the percentage of reversal of the increase in tone produced by the contractile agonists. Prism nonlinear regression analysis was used to calculate EC₅₀ values (GraphPad Software Inc., San Diego, CA).

cGMP Formation in Intact Cells. Rat aortic vascular smooth muscle cells (RASMCs) were cultured exactly as described previously (Ruetten et al., 1996). Confluent cells grown in six-well plates were rinsed two times with HEPES tyrode buffer and preincubated in 1 ml of HEPES tyrode buffer containing 0.1 mM IBMX and 200 U of superoxide dismutase. Ten microliters of HMR1766 (1–10 μM), sodium nitroprusside (SNP; 3–30 μM) or the solvent DMSO was added in the presence or absence of 1 μM ODQ. After 15 min, the supernatant was removed, and the cells were quick-frozen with liquid nitrogen and stored at −20°C. For assaying cGMP, the cells were thawed, and 400 μl of 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 (Amersham, Little Chalfont, Buckinghamshire, UK).

Phosphorylated-Vasodilator-Stimulated Phosphoprotein Formation. De-endothelialized aortic rings (eight per aorta) from Wistar rats were kept individually in 2 ml of minimum essential medium with penicillin/streptomycin at 37°C for 2 h. Then, they were transferred into 2 ml of warmed (37°C) HEPES-Tyrode containing 30 μM N^G-nitro-L-arginine. After 5 min, 10 μM NS2028 or solvent (0.1% DMSO) was added to half of the rings, and after a further 10 min, solvent (0.1% DMSO), SNP (1 μM), or S3448 (3 and 30 μM) was added. After 10 min, the rings were frozen and homogenized in liquid nitrogen. SDS-polyacrylamide gel electrophoresis and electroblotting were performed as described previously (Mulsch et al., 2001). Immunoblotting was performed with a mouse monoclonal antibody (16C2) specific for phosphorylated-vasodilator-stimulated phosphoprotein (P-VASP) at Ser239, as described previously (Mulsch et al., 2001).

Determination of Protein Content. Protein pellets were dissolved in 100 μl of 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 as standard.

Hemodynamic Studies in Anesthetized Pigs. Randomized male pigs (German landrace, 25–35 kg body weight, *n* = 5 per group) were anesthetized with pentobarbital sodium (intravenous bolus of 19–21 mg/kg, followed by a continuous infusion of 16–19 mg of pentobarbital/kg/h i.v. to maintain anesthesia) and artificially ventilated with room air and oxygen. Systolic and diastolic blood pressures and heart rate were continuously monitored with tip catheters

(Millar PC 350; Millar Instruments Inc., Houston, TX) 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 5 ml of polyethylene glycol 400. Four treatment groups were evaluated: placebo (polyethylene glycol 400), HMR1766 3 mg/kg, ODQ 1 mg/kg, and the combination HMR1766 3 mg/kg plus ODQ 1 mg/kg. The hemodynamic parameters were monitored for at least 120 min.

Statistical Analysis. Results are expressed as mean ± S.E.M., if not indicated otherwise. The EC₅₀ value for each experiment was obtained by logit transformation. One-way analysis of variance was used for comparisons of vascular responses and sGC activity. *P* < 0.05 was considered significant. The Bonferroni correction was applied for comparison of multiple means.

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 sGC isolated from bovine lung. Two compounds, S3448 and HMR1766 free acid (Fig. 1), that were obtained in an extensive chemical optimization program (data presented at the Gordon Conference on Medicinal Chemistry, New London, CT, 2004) induced a concentration-dependent 25-fold stimulation with EC₅₀ values 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 (EC₅₀ = 1.54 μM) and even less effective (13.5-fold maximal increase). Stimulation of sGC by a submaximally activating concentration of DEANO (1 μM) was additive to activation by HMR1766 throughout the entire concentration-response relationship (Fig. 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 (Fig. 2B). *V_{max}* values were 5.1 ± 0.9 nmol/mg/min under basal conditions and 52.9 ± 11.4 nmol/mg/min under stimulated conditions. The extrapolated linear graphs intersected above the *x*-axis, indicating mixed-type activation kinetics (change in *V_{max}* and *K_M* values).

Effect of sGC Heme-Iron Oxidation State on Activation by S3448. To assess the influence of the oxidation state of the sGC heme on activation by S3448, the activity of 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 was significantly increased in the presence of NS2028 by 70% (Fig. 3A). This effect was not restricted to the purified bovine

TABLE 1

Effect of anthranilic acid derivatives and DEA-NONOate (DEANO) on enzymatic activity of partially purified sGC from bovine lung, determined by PP_i formation from GTP by 0.17 μg/ml sGC. Maximal enzyme activity (*V_{max}*) is expressed as -fold stimulation of basal activity. Mean values ± S.E.M. of *n* experiments, as indicated in parentheses, are shown.

Compound	EC ₅₀	<i>V_{max}</i> -Fold Basal
	μM	
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)

enzyme but was even more pronounced with sGC present in protein extract from human corpus cavernosum. As shown in Fig. 3B, the approximately 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, probably represents a difference in relative abundance of ferrous versus 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 preincubated with S34348 is sensitive to dilution, according to the law of mass action. Therefore, NS2028-treated enzyme from bovine lung (Fig. 4A) or human corpus cavernosum (Fig. 4B) was incubated with and without 10 μM S3448 for 5 min at RT. Thereafter, the mixtures were diluted 10-fold, and sGC activity was determined, with final S3448 concentration adjusted to either 1 or 10 μM . As illustrated in Fig. 4B, the activation of sGC in human tissue was independent of the S3448 concentration at preincubation (■) and depended entirely on S3448 concentration during the activity assessment (Fig. 4B, □). This finding indicates a complete and rapid reversibility of sGC activation in human corpus cavernosum.

The purified enzyme from bovine lung behaved slightly different from the crude human enzyme. After dilution of S3448-preincubated bovine lung sGC, a lower enzyme activity was detected compared with enzyme preincubated with solvent (Fig. 4A). This finding indicates that activation of the heme-oxidized purified bovine enzyme is also rapidly reversible, but that in addition, preincubation with S3448 decreases subsequent activation of the enzyme by S3448. How-

ever, when preincubation was performed in the absence of ODQ, no decrease in subsequent activation was seen (data not shown).

In further dilution experiments with HMR1766-preincubated purified bovine lung enzyme, a curvilinear decrease of cGMP production was observed, in contrast to a linear decrease observed with only solvent-preincubated 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), 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 Fig. 5A, S3448 and SNP were approximately equipotent activators of the purified bovine enzyme, stimulating basal sGC activity maximally by approximately 17- and 14-fold. In contrast, with the crude human enzyme, nearly 8-fold higher activity was elicited by SNP compared with S3448 (Fig. 5B).

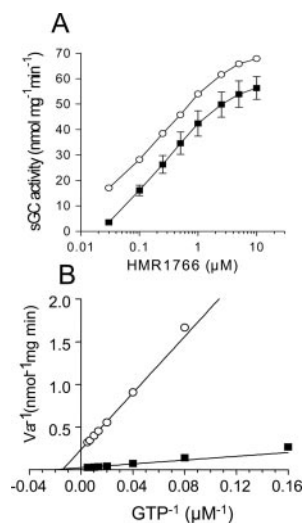


Fig. 2. A, the concentration-response curve for HMR1766-induced stimulation of sGC activity (0.5 μg of sGC partially purified from bovine lung, 60 min, 25°C, PP_i assay) was obtained in the absence (■) or presence of 1 μM DEA-NONOate (□). Symbols represent mean values \pm S.E.M. from $n = 6$ (HMR1766) 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 HMR1766 free acid (■, 0.3 μM)-stimulated sGC activity (V_a). sGC activity was determined with 0.85 μg of sGC and different concentrations of GTP (6–200 μM). B, summarized data from three independent experiments are shown.

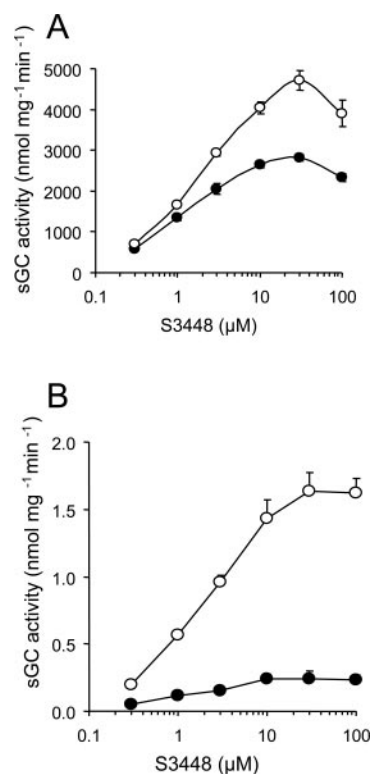


Fig. 3. Influence of NS2028 on S3448-dependent activation of sGC purified from bovine lung (20–40 ng; A) and crude sGC of human corpus cavernosum extract (10 μg ; B). The enzymatic formation of [³²P]cGMP was started by the addition of enzyme diluted in assay buffer solution (see *Materials and Methods*) to a reaction mixture containing [α -³²P]GTP (200 μM), S3448 (0.3–100 μM), 2% DMSO (●), or 10 μM (A) or 100 μM NS2028 (B) (○), and the reaction proceeded for 10 min at 37°C. [³²P]cGMP was then isolated, quantified by scintillation counting, and the specific sGC activity calculated (nanomoles of cGMP formed per milligram of protein per minute of incubation time). Mean values \pm S.E.M. from four experiments were performed in duplicate.

The porphyrin stimulated the basal activity of the bovine sGC by 6.6-fold (Fig. 5A) and the human enzyme to only 30% of the activity seen with S3448 (Fig. 5B). In the 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, whereas 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 Fig. 5, A and B, 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. 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 (Fig. 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 the activation of sGC by HMR1766/S3448. PIX (5–100 nM) induced a 7.7- to 9.7-fold increase in basal activity of purified bovine lung sGC (Fig. 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 (Fig. 6A) and decreased the maximal activation achieved by 10 μ M HMR1766 free acid. A similar result was obtained with the purified enzyme from bovine lung, which

was assessed in the presence of 10 μ M NS2028 to achieve complete heme oxidation. PIX (1 μ M) 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 μ M) by 45% (Fig. 6B). In accordance with this finding, the activation of the NS2028 (100 μ M)-exposed human enzyme from corpus cavernosum by S3448 was significantly inhibited by 1 μ M PIX to 57% of its maximal activity (Fig. 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. Heme-depleted bovine sGC, which showed a $90 \pm 5\%$ reduction of maximal activation by 100 μ M SNP (data not shown), was even more activated by S3448 than the native heme-intact enzyme (Fig. 7A). This activation by S3448 was completely abolished in the presence of 1 μ M PIX, which stimulated basal activity of the heme-depleted enzyme by 60-fold (Fig. 7B).

Because these findings would suggest that S3448 also activates the heme-free enzyme, we tested the influence of a short-term addition of Tween 20 (0.5%) to the ongoing sGC activity test 20 min after the start of the reaction, and allowed cGMP formation to proceed for a further 40 min. As shown in Fig. 7C, the addition of Tween 20 immediately

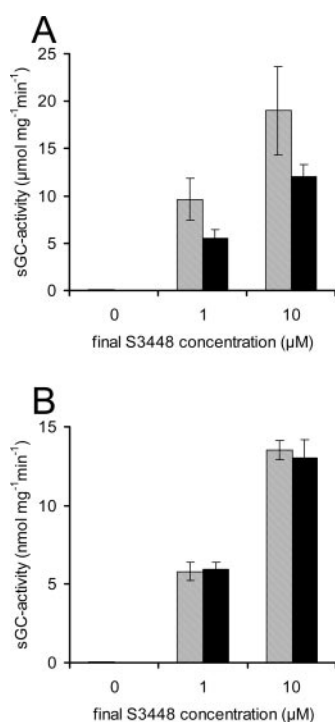


Fig. 4. Reversibility of S3448-induced activation of NS2028-treated sGC from bovine lung (A) and human corpus cavernosum (B). The enzyme was incubated with 10 μM (A) or 100 μM NS2028 (B) for 10 min at 4°C and then with (■) or without 10 μM S3448 (□) for further 5 min at RT. Thereafter, the mixtures were diluted 10-fold into a solution for the 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. Shown are mean values \pm S.E.M. from $n = 4$.

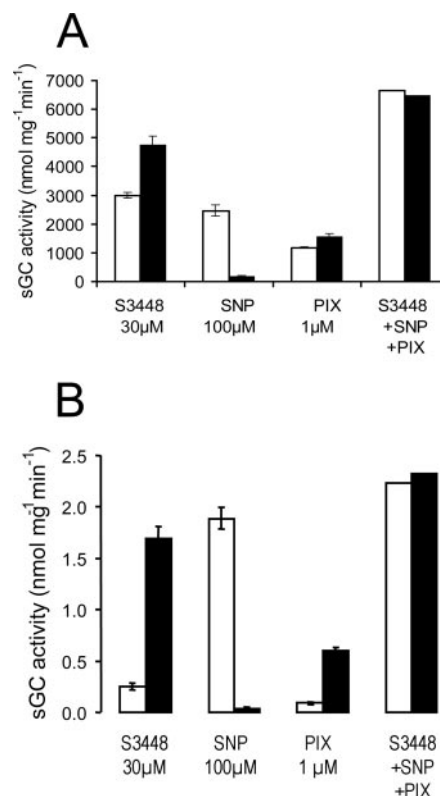


Fig. 5. Influence of NS2028 on sGC activation by S3448 (30 μM), SNP (100 μM), and PIX (1 μM). The activity of sGC from bovine lung (A) and human corpus cavernosum (B) was determined as described in Fig. 3 in the absence (□) or presence (■) of NS2028. The last pair of columns represents the calculated sum of sGC activities (S3448 + SNP + PIX). Shown are mean values \pm S.E.M. from $n = 4$.

stopped cGMP formation by S3448 (1 μ M)- and DEANO (10 μ M)-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 μ M)- and S3448 (10 μ M)-induced activation of the native bovine sGC in a concentration-dependent manner (IC_{50} = 0.6 μ M), reaching almost complete inhibition at 10 μ M (Fig. 7D). Zinc protoporphyrin IX also inhibited NO-dependent sGC activation by SNP, albeit with 10-fold lower potency (Fig. 7D).

Effect of HMR1766 on cGMP Formation in Smooth Muscle Cells. In cultured RASMCs, HMR1766 elicited a concentration-dependent increase in cGMP formation, which (at 10 μ M) rose to approximately 107-fold of the solvent value (Table 2). In the presence of 1 μ M ODQ, basal cGMP levels were decreased by half, and SNP-induced cGMP formation, which was comparable with 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 Protein Kinase I by S3448. The phosphorylation of the ubiquitous substrate of cGMP-dependent protein kinase I, vasodilator-stimulated

phosphoprotein (VASP), at Ser239 is a reliable biochemical monitor of cGMP-dependent protein kinase I activity in cells and tissues (Oelze et al., 2000). As illustrated by the representative Western blot (Fig. 8), incubation of rat aortic tissue with SNP (1 μ M) or S3448 (3 and 30 μ M) elicited a concentration-dependent phosphorylation of VASP, as measured by a specific antibody for P_{Ser239}-VASP (Oelze et al., 2000). Two P-VASP-positive peptide bands were detected at approximately 45 and 48 kDa, probably representing mono- and diphosphorylated protein (Oelze et al., 2000). P-VASP formation in response to SNP was completely blocked by the addition of NS2028 (10 μ M), whereas P-VASP formation induced by S3448 was significantly enhanced. According to a densitometric evaluation of both immunoreactive bands (Fig. 8), the ratio of SNP (1 μ M)- versus S3448 (30 μ M)-induced VASP phosphorylation decreased from 2:1 to 1:5.

Vasorelaxation Effects of S3448 and HMR1766. S3448 elicited a concentration-dependent relaxation in precontracted endothelium-denuded rings from RA, PCA, and strips from HCC (Fig. 9A). S3448 was the most potent in PCA (EC_{50} = 1.2 μ M), less so in RA (EC_{50} = 5.9 μ M), and least in HCC (10 μ M). Complete relaxation was achieved by 30 μ M S3448 in PCA and 100 μ M in RA, whereas we could not achieve more than 80% relaxation with HCC because of solubility/solvent problems with S3448 at concentration exceeding 100 μ M. The S3448-elicited relaxation of RA was significantly inhibited by pretreatment of the vessels with 3 μ M zinc-protoporphyrin IX (Fig. 9A, \blacktriangle). One-hour preincubation of aortic rings with S3448 (30 μ M) followed by 30-min wash-out had only a slight effect on the subsequent concentration-response curve for S3448-induced relaxation (maximal relaxation, 88–100%; Fig. 9B). This finding demonstrates the absence of in vitro tachyphylaxis to S3448. In contrast, preincubation of rat aortic rings with ODQ (10 μ M, 50 min) significantly shifted the concentration-relaxation curve for HMR1766 to the left (EC_{50} = 0.4 versus 1.2 μ M; p < 0.05) (Fig. 9C).

Effect of HMR1766 on Blood Pressure. In anesthetized pigs with baseline systolic (126 \pm 5 mm Hg) blood pressure, intravenous bolus application of placebo or ODQ (1 mg/kg) induced weak changes in systolic blood pressure (Fig. 9D). An intravenous dose of 3 mg/kg HMR1766 caused a long-lasting decrease in systolic blood pressure (Fig. 9D). The HMR1766-induced hypotension was potentiated by the simultaneous injection of 1 mg/kg ODQ and developed to a maximal decrease of 49 mm Hg within 15 min after administration. The duration of ODQ potentiation lasted for 120 min. Qualitatively similar effects were observed on diastolic blood pressure (data not shown). The heart rate (baseline 94 \pm 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 that 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 inhi-

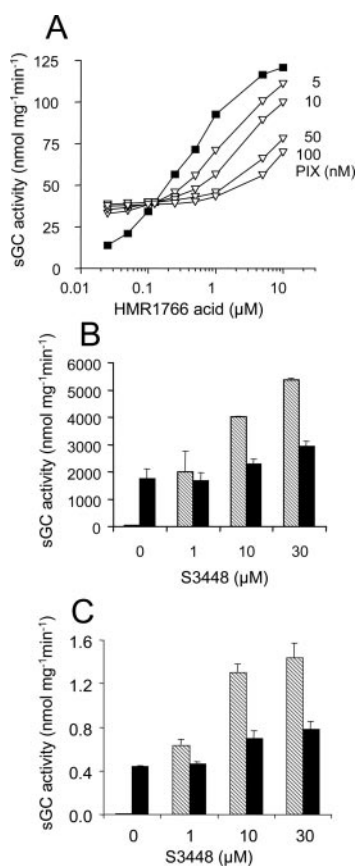


Fig. 6. Interference of PIX with activation of sGC by HMR1766 (A) acid and S3448 (B and C). A, the activity (PP_i formation from GTP) of partially purified bovine lung sGC (0.17 μ g/ml) was assessed in the presence of different concentrations of PIX (5–100 nM) and HMR1766 acid, as indicated. A representative experiment is shown. B, the activation of NS2028 (10 μ M)-treated bovine lung sGC (25 ng) by S3448 (1–30 μ M) was assessed in the absence (□) or presence (■) of 1 μ M PIX. Shown are mean values \pm S.E. of n = 2. C, the activation of NS2028 (100 μ M)-treated human corpus cavernosum sGC (10 μ g) by S3448 (1–30 μ M) was assessed in the absence (□) or presence (■) of 1 μ M PIX. Values shown are the mean \pm S.E. of n = 2.

bition 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 antihypertensive and antianginal compounds, the so-called NO donors/nitrovasodilators (Feelisch, 1998). However, their therapeutic value has been challenged recently by the finding that their long-term use is associated with increased superoxide formation (Munzel et al., 2000). Thus, the use of NO donors can give rise to the formation of the deleterious peroxynitrite (ONOO^-), as has been shown in nitrate-tolerant states, for instance (Munzel et al., 2000). 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 pharmacological activity of two lead compounds of a new class of activators of sGC, HMR1766 and S3448 (Fig. 1). Both compounds stimulated the activity of purified sGC from bovine lung and 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 protein kinase 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 anesthetized 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). Because 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 fer-

ric heme-redox form of sGC. It furthermore suggested that heme-oxidized enzyme was present to a small degree in isolated enzyme preparations and crude tissue homogenates. Closer examination of UV-visible 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 (Fig. 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, because recently, Stasch and colleagues (2002) reported similar findings with their new sGC activator BAY 58-2667. 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 BAY 58-2667 with protoporphyrin IX, by competition experiments, and using purified heme-depleted sGC, these authors concluded that BAY 58-2667 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), although it may also activate heme-intact forms by the 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 (Fig. 6) and that zinc-protoporphyrin IX was a more potent inhibitor of HMR1766/S3448-dependent than of NO-dependent sGC activity (Fig. 7D). Thus, the possibility exists that HMR1766/S3448 activate the heme-free enzyme.

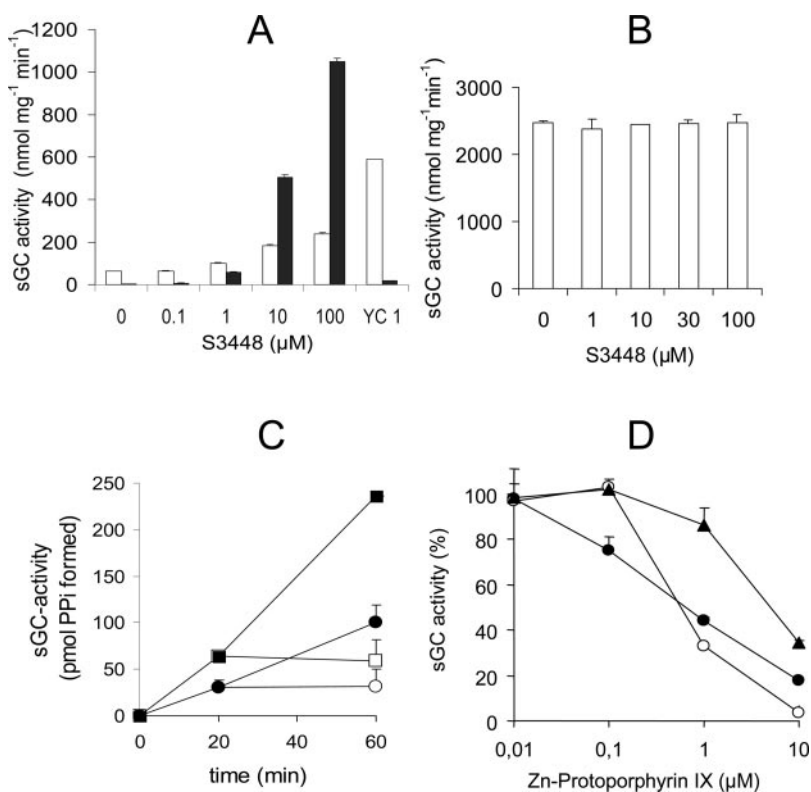


Fig. 7. Effect of heme depletion and zinc-protoporphyrin IX on the activation of sGC by S3448 and HMR1766. A, activation of native (□) and heme-depleted (0.5% Tween 20-treated; ■) bovine lung sGC (0.4 ng) by S3448 and YC-1 (100 μM). Values shown are the mean ± S.E. of $n = 2$. B, activation of heme-depleted bovine lung sGC (50 ng) by S3448 was assessed in the presence of 1 μM PIX. Values shown are the mean ± S.E. 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 (●, ■) the addition of 0.5% Tween 20 after 20 min. Values shown are the mean ± S.E. of $n = 3$. D, inhibition by zinc protoporphyrin IX of activation of bovine lung sGC by HMR1766 free acid (10 μM; ●), S3448 (10 μM; ○), and SNP (100 μM; ▲).

However, evidence against this mechanism of action was provided by the finding that HMR1766-induced cGMP formation immediately ceased after a short-term depletion of the heme by addition of Tween 20 to an ongoing sGC enzyme reaction (Fig. 7C). Furthermore, S3448, even at 100 μ M, was not able to alter the activity of heme-depleted sGC in the presence of maximally activating concentration of protoporphyrin IX (Fig. 7B), although this was still possible with NS2028-treated enzyme (Fig. 6, B and C). Our findings are best explained by the assumption that the ODQ/NS2028 treatment to a certain extent also leads to the 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 Fig. 5, A and B. Furthermore, it is conceivable that the ferric heme will exchange more easily with protoporphyrin IX than with the ferrous enzyme, which explains the competitive antagonism of protoporphyrin IX with regard to HMR1766/S3448-supported sGC activity. Indeed, exchange of the ferric heme with protoporphyrin IX and other porphyrins has been demonstrated previously (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

TABLE 2

Effect of HMR1766 and SNP on cGMP concentrations (mean \pm S.E., $n = 3$) in RASMCs in the absence ($-$ ODQ) and presence ($+$ ODQ) of 1 μ M ODQ

The cells were exposed to sGC activators for 10 min in the presence of 0.1 mM IBMX.

Compound	Concentration μ M	cGMP	
		$-$ ODQ <i>pmol/mg protein</i>	$+$ ODQ <i>% of $-$ODQ</i>
Solvent		0.80 \pm 0.08	53
HMR1766	1	14.70 \pm 1.00	445
HMR1766	3	46.50 \pm 2.32	451
HMR1766	10	85.70 \pm 2.78	485
SNP	3	45.50 \pm 4.20	1.4
SNP	10	43.00 \pm 3.57	3.2
SNP	30	70.10 \pm 3.61	2.6

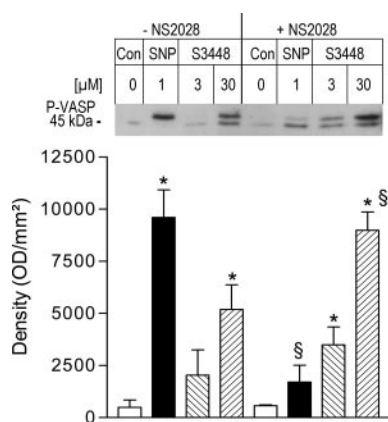


Fig. 8. S3448-elicited phosphorylation of VASP in rat aorta. De-endothelialized rat aortic rings were incubated in the absence and presence of NS2028 (10 μ M) and after 10 min were stimulated for 10 min with either solvent (0.1% DMSO; "Con"), SNP, or S3448, as indicated. Tissues were homogenized and subjected to Western blot analysis for P-VASP. The representative immunoblot shows the two immunoreactive bands of P-VASP at \sim 45 kDa. The column diagram below represents a densitometric evaluation (mean \pm S.E.M.) of three different experiments performed with rings from three rats. *, $P < 0.05$ versus control; §, $P < 0.05$ versus NS2028.

activators exhibited potent pharmacological activity in vascular cells and tissues, and even living animals. This observation provides the 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 (Table 2). 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, whereas HMR1766-induced cGMP formation increased by 5-fold. According to this observation, the ferric enzyme would account for only 20% of the total sGC activity present in cultured rat aortic smooth muscle cells. We now have a 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 that is not sensitive to SNP. A similar estimate for the proportion of HMR1766/S3448- versus 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 homog-

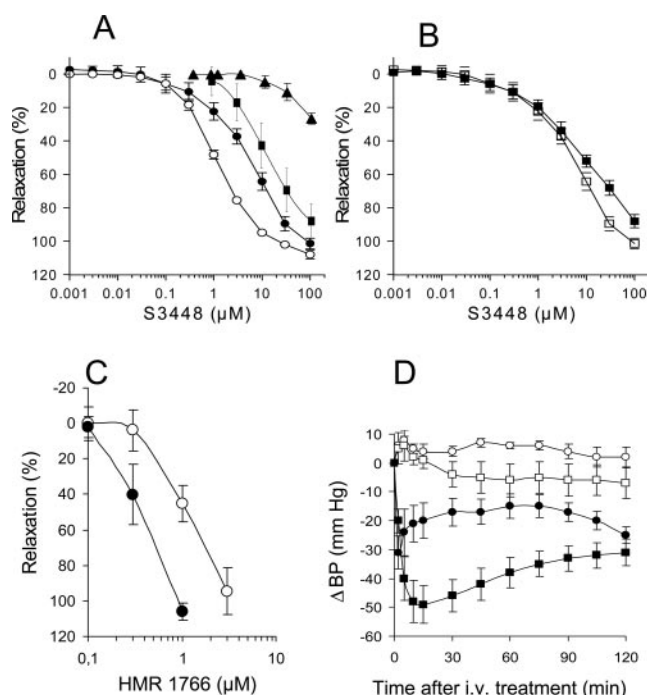


Fig. 9. Vasodilatory and hypotensive effects of S3448. A, concentration-dependent relaxation by S3448 of PE (1 μ M)-contracted endothelium-denuded RA (●), U46619 (1 μ M)-contracted porcine coronary artery (○), and noradrenaline (3–7 μ M)-contracted human corpus cavernosum (■). The relaxation of RA was completely inhibited by 3 μ M zinc-protoporphyrin added 30 min before PE (▲). Data show are the mean \pm S.E. of three to four different vessels from either species. B, the effect of 60-min in vitro pretreatment with either solvent (□) or 30 μ M S3448 (■) on relaxation of PE (1 μ M)-contracted rat aorta by S3448. Mean \pm S.E. of up to eight rings from three rats. C, effect of 50 min in vitro pretreatment with either solvent (○) or 30 μ M ODQ (●) on relaxation of PE (0.1 μ M)-contracted rat aorta by HMR1766. Mean \pm S.E. of six rings from two rats. D, effect of HMR1766 on systolic blood pressure in pentobarbital anesthetized minipigs. HMR1766 (●) 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 five pigs. PEG/placebo (○)- and ODQ (□)-treated minipigs served as control groups. Data are given as the difference between the placebo (126 \pm 2 mm Hg), ODQ (126 \pm 5 mm Hg), HMR1766 (116 \pm 6 mm Hg) and HMR1766/ODQ (131 \pm 6 mm Hg) baseline pressures.

enized human corpus cavernosum (Fig. 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 intravenously by 3 mg/kg HMR1766 (Fig. 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-protoporphyrin IX, supporting the concept that the ferric enzyme accounted for vasorelaxation by both compounds. Similar antiplatelet effects of HMR1766 observed in vitro and in vivo will be published in a future article (M. Koglin, U. Schindler, M. Just, A. Kannt, K. Breit-schopf, H. Strobel, and S. Behrends, 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 article, Witte and collaborators (2004) showed that HMR1766 significantly activated sGC assayed in vitro in internal mammary artery species from patients with coronary artery disease (with and without diabetes). 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 patients and therefore should find broad application in preclinical 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 observations).

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 particularly be beneficial in cardiovascular disease states associated with oxidative stress.

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