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Cardiovascular actions of a novel NO-independent guanylyl cyclase stimulator, BAY 41-8543: *in vivo* studies

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1 BAY 41-8543 is a novel non-NO-based stimulator of sGC. This study investigates the acute effects of BAY 41-8543 on haemodynamics in anaesthetized rats and dogs, its long-term effects in conscious hypertension rat models and its antiplatelet effects.

2 In anaesthetized dogs, intravenous injections of BAY 41-8543 (3–100 μg kg^{-1}) caused a dose-dependent decrease in blood pressure and cardiac oxygen consumption as well as an increase in coronary blood flow and heart rate.

3 In anaesthetized normotensive rats, BAY 41-8543 produced a dose-dependent and long-lasting blood pressure lowering effect after intravenous (3–300 μg kg^{-1}) and oral (0.1–1 mg kg^{-1}) administration. A dose-dependent and long-lasting decrease in blood pressure was also observed in conscious spontaneously hypertensive rats with a threshold dose of 0.1 mg kg^{-1} p.o. After 3 mg kg^{-1} the antihypertensive effect lasted for nearly 24 h. After multiple dosages, BAY 41-8543 did not develop tachyphylaxis in SHR.

4 BAY 41-8543 prolonged the rat tail bleeding time and reduced thrombosis in the $FeCl_3$ thrombosis model after oral administration.

5 In a low NO, high renin rat model of hypertension, BAY 41-8543 prevented the increase in blood pressure evoked by L-NAME and reveals a kidney protective effect. In this model, the overall beneficial effects of BAY 41-8543 manifested as both antiplatelet effect and vasodilatation were reflected in a significant reduction in mortality.

6 The pharmacological profile of BAY 41-8543 suggests therefore that this compound has the potential to be an important research tool for *in vivo* investigations in the sGC/cGMP field and it also has the potential of being a unique clinical utility for treatment of cardiovascular diseases.
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Keywords: Nitric oxide; soluble guanylyl cyclase; cyclic GMP; BAY 41-8543; YC-1; VASP; L-NAME; blood pressure; rats; dogs

Abbreviations: ANP, atrial natriuretic peptides; GTN, glycerol trinitrate; ISDN, isosorbide dinitrate; L-NAME, N$^{\omega}$-nitro-L-arginine methyl ester; PRA, plasma renin activity; sGC, soluble guanylyl cyclase; SHR, spontaneously hypertensive rats; VASP, vasodilator-stimulated phosphoprotein; YC-1, 3-(5′-Hydroxymethyl-2′-furyl)-1-benzylindazole

Introduction

Soluble guanylyl cyclase is a heterodimer with haeme as prosthetic group, which represents the intracellular receptor for the ubiquitous biological messenger NO. NO is a potent activator of sGC causing activation of up to 400 fold greater than basal level (Stone & Marletta, 1994). Activation of the enzyme facilitates conversion of GTP to the intracellular second messenger cyclic GMP, which mediates the majority of biological actions of NO. The NO/cyclic GMP pathway is important in many physiological processes including vasodilatation, neurotransmission and platelet aggregation (Moncada *et al*., 1991; Furchgott, 1999; Ignarro, 1999; Murad, 1999). Due to its ubiquitous nature, the pathogenesis of various disease states, especially of the cardiovascular system, has been linked to inappropriate activation of sGC (Hobbs, 2000). Activators of sGC are therefore very desirable as both pharmacological tools to probe the NO-cyclic GMP pathway and as potential therapeutic targets.

The best studied class of sGC activators are organic nitrates which mimic the action of endogenous NO by bioconversion to NO or NO-related compounds (Feelisch, 1998). Organic nitrates have been used for decades as treatment for angina pectoris, however, the major drawback of this therapy is the development of tolerance upon prolonged use and the absence of a clinically relevant antiplatelet activity. Therefore, there is an obvious need for novel activators of sGC that overcome these problems of organic nitrates. Recently, an indazole derivative, YC-1, has been described which stimulates sGC directly *via* a distinct mechanism and sensitizes the enzyme towards its native activator NO (Ko *et al*., 1994; Wu *et al*., 1995; Friebe *et al*., 1996; Mülsch *et al*., 1997; Hoenicka *et al*., 1999). Further studies demonstrated an effect of YC-1 on prevention of venous thrombosis in mice (Teng *et al*., 1979), on vasodilation of aortic rings (Mülsch *et al*., 1997), and a

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blood pressure lowering effect after intravenous administration of high doses (Rothermund *et al*., 2000). However, YC-1 has recently been shown also to act as a non-specific phosphodiesterase inhibitor (Galle *et al*., 1999; Friebe *et al*., 1998); inhibition of cyclic GMP metabolizing PDEs would be expected to increase the level of cyclic GMP produced and enhance the biological effects of this compound.

We used YC-1 as a chemical lead structure and selected BAY 41-8543 as the most promising compound with respect to potency, specificity as well as oral activity out of a series of around two thousand newly synthesized derivatives (Stasch *et al*., 2002; Straub *et al*., 1998; 2001a). The present study describes the haemodynamic properties of the novel sGC stimulator, BAY 41-8543, in different animal models and dogs after intravenous administration. In addition, the blood pressure lowering effect of BAY 41-8543 in conscious SHR after multiple dosing is presented. The inhibition of $FeCl_3$ induced thrombosis formation and the prolongation of rat tail bleeding time was used to show an antiplatelet activity of the compound after oral administration. From *in vitro* studies, BAY 41-8543 is more than two orders of magnitude more potent than YC-1 and devoid of any PDE inhibitory activity (Stasch *et al*., 2002). Since we have observed a synergism between BAY 41-8543 and NO on cyclic GMP production on purified sGC, in endothelial cells, and platelets (Stasch *et al*., 2002) the question arised whether *in vivo* administration of BAY 41-8543 also potentiates NO-induced vasodilatory effects. In addition, the overall beneficial effects of BAY 41-8543 manifested as both antiplatelet effect and vasodilatation, were investigated regarding development of blood pressure, kidney protection and mortality in L-NAME treated TG(mRen2)27 rats in a long-term trial.

Materials

Substances

BAY 41-8543 (2-[1-[(2-fluorophenyl)methyl]-1H-pyrazolo[3,4-b]pyridin-3-yl]-5(4-morpholinyl)-4,6-pyrimidinediamine) was synthesized as described (Straub *et al*., 1998).

Haemodynamics in anaesthetized dogs

Studies were performed on anaesthetized dogs of either sex (body weight between 20–30 kg). Anaesthesia was initiated by slow intravenous injection of 25 mg kg^{-1} sodium thiopental (Trapanal®, Byk Gulden, Konstanz, Germany). The anaesthesia was continued and maintained throughout the experiment by continuous infusion of 0.04 mg kg^{-1} h^{-1} fentanyl (Fentanyl®, Janssen, Neuss, Germany) and 0.25 mg kg^{-1} h^{-1} droperidol (Dihydrobenzperidol^R, Janssen, Neuss, Germany). During this anaesthesia, heart rate is as low as 35–40 beats min^{-1} due to increased vagal tone. Therefore, a parasympathetic blockade was achieved by intermittent injections of atropine (0.1 mg per animal) (Atropinsulfat^R, Eifelfango, Bad Neuenahr, Germany). After intubation the animals were artificially ventilated at constant volume (Engström^R 300, Engström, Sweden) with room air enriched with 30% oxygen to maintain an end-tidal CO_2 concentration of about 5% (Normocap^R, Datex, Finland). For measurement of cardiovascular parameters the following catheters were implanted: a tip catheter for recording of left ventricular pressure was inserted into the ventricle *via* the carotid artery (PC350, Millar Instruments, Houston, TX, U.S.A.), a hollow catheter was inserted into the femoral artery for recording of arterial blood pressure, and connected to a strain gauge (type 4-327-1, Telos Medical, Upland, CA, U.S.A.), two venous catheters were inserted into either femoral vein and one additional catheter into a forearm vein for application of the anaesthetic and drugs, respectively and an oxymetry catheter for recording of oxygen saturation was inserted into the coronary sinus *via* the jugular vein (Schwarzer IVH4, München, Germany). After a left-sided thoracotomy the ramus circumflexus of the left coronary artery (LCX) was freed from connective tissue and an electromagnetic flow probe (Gould Statham, Oxnard, CA, U.S.A.) was applied for measurement of coronary blood flow. Arterial blood pressure, electrocardiogram (lead II), left ventricular pressure, first derivative of left ventricular pressure (dP/dt), heart rate, coronary blood flow, and oxygen saturation in the coronary sinus were continuously recorded on a pen recorder (Brush, Gould, Cleveland, OH, U.S.A.). The maximum of dP/dt was used as measure of left ventricular contractility (dP/dt^{max}). After completion of the instrumentation an interval of 60 min was allowed for stabilization before BAY 41-8543 was intravenously applied as bolus injections. Care was taken that all measured cardiovascular parameters had returned to control level before injection of the next dose. BAY 41-8543 was dissolved in a solution of glycerol/water/polyethylenglycol 400 (60/100/949 = $g/g/g^{-2}$). Each dose of BAY 41-8543 was tested at least three times in different animals. The order of injection of the different doses of BAY 41-8543 was randomized in each animal.

Interaction with organic nitrates in anaesthetized dogs

Two sets of experiments were performed to study a possible interaction between BAY 41-8543 and GTN: In the first set, initially a cumulative dose response curve was established for GTN with three successive iv bolus injections (0.5, 1 and 3 $\mu g\ kg^{-1}$) each separated by 30–50 min. Care was taken that all measured cardiovascular parameters had returned to control level before injection of the next dose. Then BAY 41-8543 was intravenously applied as a continuous infusion over 60–120 min at a rate of 3 $\mu g\ kg^{-1}\ min^{-1}$ (0.2 ml min^{-1}). During this infusion of BAY 41-8543, the cumulative dose response curve for GTN was repeated with the same three doses as before. The second set of experiments was similar to the first one, except that the decrease in blood pressure during the infusion of BAY 41-8543 was counteracted by short periods of infusion of noradrenaline during the bolus injections of GTN: Blood pressure was raised to the control level for short periods of 3–4 min by a concomitant infusion of noradrenaline and the respective dose of GTN was injected. The infusion rate of noradrenaline was adjusted in each dog to give the same level of arterial blood pressure as before the infusion of BAY 41-8543, it varied between 0.2 and 0.3 $\mu g\ kg^{-1}\ min^{-1}$. BAY 41-8543 was dissolved as described above. GTN was applied as an aqueous solution. Noradrenaline (Arterenol injection solution 1 : 1000® Hoechst, Frankfurt, Germany) was diluted in saline for the short infusions during infusion of BAY 41-8543.

Haemodynamics in anesthetized rats

Male Wistar rats weighting 300–350 g (Harlan Winkelmann, Borchen, Germany) were anaesthetized with thiopental (Nycomed, Munich, Germany) 100 mg kg^{-1} i.p. A tracheotomy was performed and catheters were inserted into the femoral artery for blood pressure and heart rate measurements (Gould pressure transducer and recorder, model RS 3400) and into the femoral vein for substance administration. The animals were ventilated with room air and their body temperature was controlled. BAY 41-8543 was administered orally as a solution in Transcutol®/Cremophor® EL/H_2O (10/20/70 = v/v/v) gavage. For intravenous administration BAY 41-8543 was dissolved in a solution of Transcutol®/Cremophor® EL/physiological saline (10/10/80 = v/v/v).

Haemodynamics in conscious SHR

Female conscious SHR (Moellegaard, Denmark, 220–290 g) were equipped with implantable radiotelemetry, and a data aquisition system (Data Sciences, St. Paul, MN, U.S.A.), comprising a chronically implantable transducer/transmitter unit equipped with a fluid-filled catheter was used. The transmitter was implanted into the peritoneal cavity and the sensing catheter was inserted into the descending aorta.

Single administration of BAY 41-8543 was performed as a solution in Transcutol®/Cremophor®/H_2O (10/20/70 = v/v/v) given orally by gavage. The animals of control groups only received the vehicle. Before treatment mean blood pressure and heart rate of treated and untreated control groups was in the range of 131–142 mmHg and 279–321 beats min^{-1}, respectively.

Multiple dosing in conscious SHR

BAY 41-8543 or vehicle was orally administered once daily over 4 days. All animals received only the vehicle on day 5. Before treatment mean blood pressure and heart rate of treated and control groups were 132 or 135 mmHg and 297 or 320 beats min^{-1}, respectively. Pressure pulses were recorded from each individual for 10 s every 5 min. Data were grouped to provide 15 min means. Blood pressure monitoring was performed continuously over 24 h. Data acquisition was started 2 h before drug administration.

Six animals were used for each group and mean values of arterial blood pressure and heart rate were calculated for each group. Effects were calculated as per cent change of mean values from the baseline level. Baseline level of each group was calculated as mean value from the 2 h pretreatment period.

Rat tail bleeding time

Male Wistar rats weighing 280–320 g (Harlan Winkelmann, Borchen, Germany), were orally treated with BAY 41-8543 (0.1, 0.3 and 1.0 mg kg^{-1}), acetylsalicylic acid (30 mg kg^{-1}) or vehicle (Transcutol®/Cremophor® EL/H_2O; 10/20/70 = v/v/v). 50 min after the oral dosing rats were anesthetized with thiopental (Nycomed®, Munich, Germany) 100 mg kg^{-1} i.p. and placed in a tube holder with the tail allowed to protrude. After additional 20 min, the terminal 2 mm tip of the tail was removed with a sterile razor blade, and the tail was vertically immersed into normal saline at 37°C. The bleeding time was then measured as described (Busse & Seuter, 1981).

$FeCl_3$ arterial thrombosis model in rats

Male Wistar rats weighing 180–220 g (Harlan Winkelman, Borchen, Germany) were anaesthetized with xylazine (12 mg kg^{-1} i.p.) followed by ketamine hydrochloride (50 mg kg^{-1} i.p.). After exposure of the left common carotid artery vascular damage was produced by placing a piece of filter paper (8 × 6 mm) placed on a strip of parafilm under the vessel and adding 20 μl of 10% $FeCl_3$ (in 1N HCl) onto the filter paper according to a method described previously (Kurz *et al*., 1990). The filter paper was removed after 3 min and the vessel was rinsed with 0.9% NaCl. The carotid artery was removed 15 min after the application of the filter paper. The thrombus was withdrawn and weighed immediately. Bay 41-8543 (3 mg kg^{-1} p.o.) was given 75 min before damage of the vessel as a solution in EtOH/ Solutol®/H_2O (10/40/50 = v/v/v). The animals of the control group received the vehicle. Ten animals were used for each group.

Long-term study

Twenty-seven male renin transgenic rats (TGR(mRen2)27) were bred in house and were housed under controlled conditions of light and temperature. At the age of 18 weeks, the animals were randomized in two groups, a control group and a group treated with BAY 41-8543. Rats of both groups were given L-NAME in the drinking water (500 mg l^{-1}), rats of the BAY 41-8543 group additionally received 3 mg kg^{-1}. BAY 41-8543 p.o. twice daily for 5 weeks, whereas the control group received no treatment. The substance was administered as a suspension in Transcutol®/Cremophor EL/water (10/20/70 = v/v/v) by gavage. Systolic blood pressure and heart rate was measured weekly by the tail-cuff method in conscious animals in cages kept a constant temperature of 37°C. At the end of the study, the animals were sacrificed by decapitation. Blood samples were collected after decapitation into chilled tubes.

Biochemical analysis of plasma parameters

ANP The ANP in EDTA plasma was determined after extraction using C18-cartridges (Bond Elut, Varian, Horbor City, CA, U.S.A.) and a specific and sensitive radioimmunoassay kit (Biotrend, Köln, Germany).

Renin activity PRA was determined by incubation of rat EDTA plasma with phenyl-methylsulfonyl fluoride. Angiotensin I accumulated in the plasma samples during incubation for 1 h at 37°C at pH 6.0 and was measured using a commercial radioimmunoassay kit (Sorin Biomedia, Saluggia, Italy).

Aldosterone Plasma aldosterone was also determined with a radio-immunoassay kit from Sorin Biomedia.

Creatinine and urea These plasma parameters were determined by methods described previously (Gutman & Bergmeyer, 1974).

Cyclic GMP For determination of cyclic GMP an equal volume of 10% trichloroacetic acid was added to the samples. After an incubation period of 30 min the samples were centrifuged, and the supernatant was washed four times with water-saturated ether and lyophilized. The cyclic GMP content was determined using a commercial radio-immunoassy kit (IBL, Hamburg, Germany).

Statistics

Differences were checked for significance by Student's *t*-test (one-way ANOVA) for unpaired data. All values in the tables and figures are given in the form of means ± s.e.mean if not otherwise indicated. $*P<0.05$, $**P<0.001$, $***P<0.001$ compared to values in untreated controls. Chi-quadrat test was used for the mortality results.

Results

Haemodynamics in anaesthetized dogs

BAY 41-8543 was tested with intravenous bolus injections ranging from 3–100 μg kg^{-1}. Intravenous bolus injections of BAY 41-8543 caused a dose-dependent decrease in mean arterial blood pressure (Table 1, Figure 1b). The minimal effective dose for the blood pressure response was 3 μg kg^{-1} resulting in a decrease by 6 mmHg (−6.5%). The highest dose tested (100 μg kg^{-1}) caused a decrease by 25 mmHg (−26.6%). Coronary blood flow was increased (Table 1, Figure 1a): the lowest dose caused an increase by 11 ml min^{-1} (31.4%), the highest dose an increase by 32 ml min^{-1} (78.0%). Due to the increase in coronary blood flow oxygen saturation in the coronary sinus was also increased (Table 1, Figure 1d). At all doses BAY 41-8543 caused a slight increase in heart rate, most likely due to the decrease in blood pressure and subsequent activation of the baroreceptor reflex (Table 1, Figure 1c). At the dose of 3 μg kg^{-1} heart rate was increased by 10 beats min^{-1} (14.5%), at the highest dose of 100 μg kg^{-1} the increase was 23 beats min^{-1} (29.1%). At all doses BAY 41-8543 had no effect on dP/dt_{max} (Table 1). The onset of action of BAY 41-8543 was rapid and reached a maximum within 30–60 s at the highest dose. The effects lasted for several minutes, after the two highest doses for up to 5–8 min. There was no significant effect of BAY 41-8543 on left ventricular end-diastolic pressure.

Interaction with organic nitrates in anaesthetized dogs

Intravenous bolus injections of GTN (0.5 to 3 μg kg^{-1}) caused a dose-dependent, short-lasting (1–3 min) decrease in mean arterial blood pressure to absolute values as low as 53 to 62 mmHg at 3 μg kg^{-1} (mean 58 mmHg) (Figure 2a–c). Infusion of BAY 41-8543 (3 μg kg^{-1} min^{-1}) caused a steady decrease in mean blood pressure by 21 mmHg to absolute values between 65 and 70 mmHg (mean 67 mmHg) (Table 2, Figure 2a). This effect was maintained throughout the duration of the infusion (60–120 min). Intravenous bolus injections of GTN (0.5 to 3 μg kg^{-1}) in the presence of BAY 41-8543 caused a dose-dependent decrease in mean blood pressure to lower values than in the absence of BAY

Table 1 Effects of intravenous bolus injections of BAY 41-8543 on systolic blood pressure (SBP), diastolic blood pressure (DBP), mean arterial blood pressure (MAP), left ventricular contractility (dp/dt_{max}), heart rate (HR), coronary blood flow (CBF), and oxygen saturation in the coronary sinus (SO_2) in anaesthetized dogs

Dose (μg kg^{-1})	*SBP* (mmHg)		*DBP* (mmHg)		*MAP* (mmHg)		dp/dt_{max} (mmHg s^{-1})		*HR* (beats min^{-1})		*CBF* (ml min^{-1})		SO_2 (%)		*n*
	Before	*After*	*Before*	*After*	*Before*	*After*	*Before*	*After*	*Before*	*After*	*Before*	*After*	*Before*	*After*	
3	123±1.5	117±3.3	77±1.7	70±0**	92±0.6	86±1.3**	2033±120	2033±120	69±3.5	79±2.3	35±6.1	46±10.4	35±1.3	39±3.7	3
10	119±4.0	111±4.3	75±2.7	66±1.9*	90±3.1	81±2.6*	2000±158	2020±153	65±3.0	75±4.4	35±2.5	44±4.7	34±4.6	39±5.5	5
30	119±3.4	101±4.8**	78±1.1	62±1.0**	92±1.5	75±2.2***	2080±132	2060±93	73±7.0	86±9.0	36±4.8	48±4.6*	34±2.8	45±1.8**	5
100	124±5.7	94±6.5**	78±3.8	59±2.1**	94±4.4	69±3.8**	2200±235	2200±248	79±4.9	102±7.2*	41±6.8	73±16.8*	37±2.4	49±3.4**	4

The values are means ± s.e.mean. $*P<0.05$, $**P<0.01$, $***P<0.001$.

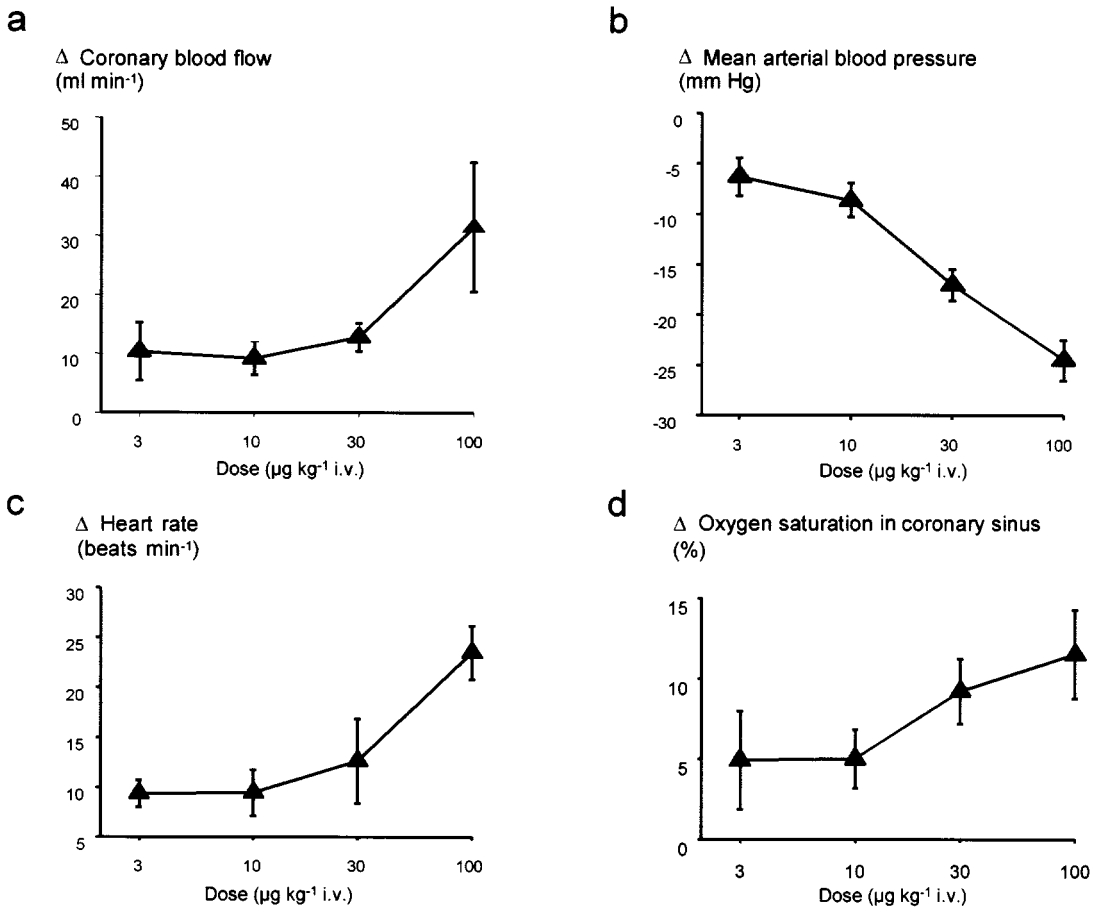


Figure 1 Dose response curve of the effects of BAY 41-8543 on coronary blood flow (a), mean arterial blood pressure (b), heart rate (c), and oxygen saturation in the coronary sinus of anaesthetized dogs (d). The graph illustrates the change in each haemodynamic parameter after i.v. bolus injection with respective to pre-drug level. Values are means ± s.e.mean. Number of animals per dose were $n=3$ at 3 μg kg^{-1}, $n=5$ at 10 and 30 μg kg^{-1}, $n=4$ at 100 μg kg^{-1}.

41-8543 (Figure 2a,c) (to a mean of 49 mmHg at 3 μg kg^{-1}). The decrease in blood pessure by GTN in the presence of BAY 41-8543, however, was always less than the algebraic sum of the decrease in the blood pressure by either compound given alone. If blood pressure was elevated to control levels during the infusion of BAY 41-8543 by short concomitant infusions of noradrenaline for the time of the intravenous bolus injections of GTN, then GTN caused a dose-dependent decrease in mean arterial blood pressure to the same absolute values between 45 and 58 mmHg at 3 μg kg^{-1} (mean 51 mmHg) as in the absence of the noradrenaline infusion (Figure 2b). Starting from a higher level the decrease in blood pressure, however, was markedly larger (Figure 2d) indicating a synergism between GTN and BAY 41-8543. There was no effect of GTN bolus injections on (dP/dt_{max}); during the infusion of BAY 41-8543 dP/dt_{max} was slightly depressed by 10–20%. Neither bolus injections of GTN nor infusion of BAY 41-8543 caused any marked effects on heart rate.

Haemodynamics in anaesthetized rats

BAY 41-8543 produced a dose-dependent and long-lasting decrease in blood pressure in anaesthetized rats (Figures 3 and 4). After intravenous administration of 0.003, 0.01, 0.03, 0.1, and 0.3 mg kg^{-1}, maximal blood pressure lowering effects of 0, −16, −35, −72 and 83 mmHg, respectively, were observed (Figure 3). The blood pressure lowering effect of BAY 41-8543 lasted longer than the observation period of 30 min. Only a minor increase in heart rate was observed (Figure 3). After oral administration of 0.1, 0.3 and 1.0 mg kg^{-1}, maximal blood pressure lowering effects of −15, −29, and −41 mmHg, respectively, were observed (Figure 4). The blood pressure lowering effects of the highest doses of BAY41-8543 lasted longer than the observation period of 120 min. Only after the highest dose of BAY 41-8543, a slight increase in heart rate was observed (Figure 4).

Haemodynamics in conscious SHR

BAY 41-8543 produced a dose-dependent and long-lasting decrease in mean blood pressure in conscious SHR (Figure 5a). A threshold effect was observed after administration of 0.1 mg kg^{-1}, when pressure dropped by 5–10% (6–13 mmHg) from initial value. A dose of 0.03 mg kg^{-1} did not influence blood pressure in comparison to control group. After 0.3, 1.0, 3.0 and 10 mg kg^{-1} mean blood pressure decreased in individual animals by 9–18% (12–24 mmHg), 14–19% (20–26 mmHg), 17–25% (24–36 mmHg) and 23–50% (21–66 mmHg), respectively. After 10 mg kg^{-1} a

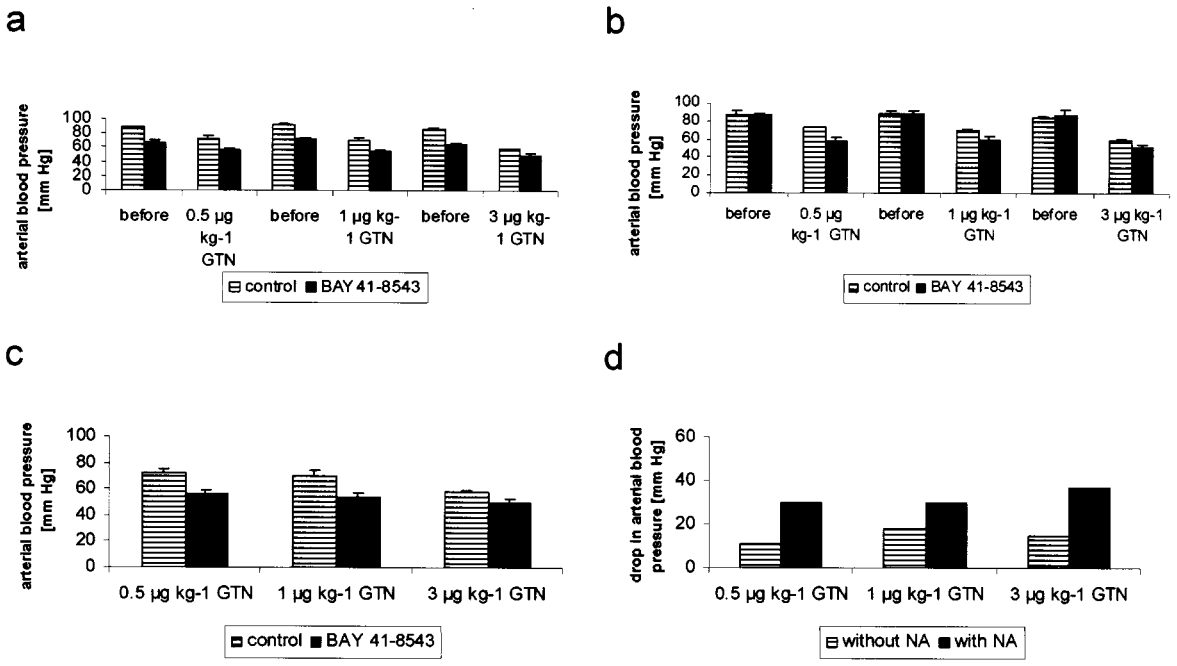


Figure 2 Effects of GTN on mean arterial blood pressure (a) during control periods and during infusion of BAY 41-8543 ($n=5$) and (b) during control periods and during simultaneous infusion of BAY 41-8543 and noradrenaline (NA) in anaesthetized dogs ($n=3$). a,b: hatched bars: without BAY41-8543, solid bars: during the infusion of BAY 41-8543. In (c) only the minimum of mean arterial blood pressure after GTN is shown in the absence (hatched bars) and the presence of BAY 41-8543 (solid bars). In (d) only the minimum of mean arterial blood pressure after GTN is shown in the presence of BAY 41-8543 without (hatched bars) and with elevation (solid bars) of blood pressure to control values. Values are taken from Table 1 and are means$\pm$s.e.mean.

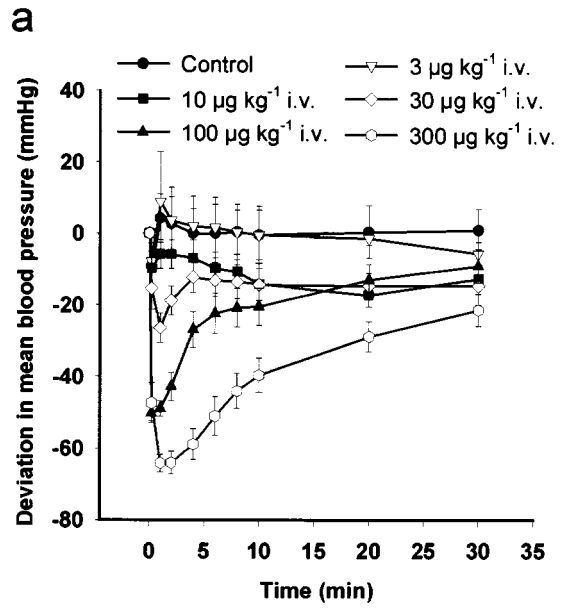
biphasic response was observed: after initial strong, but short lasting decrease (by about 50%) blood pressure stabilized after approx. 2.5 h at a markedly lower level (-23%) for more than 24 h. The onset of blood pressure lowering effect was fast with the maximum decrease achieved within the first hour after administration; it lasted for about 8 (0.1 mg kg^{-1}), 10 (0.3 mg kg^{-1}), 14 (1 mg kg^{-1}) and more than 24 h (3 and 10 mg kg^{-1}). In the vehicle treated group blood pressure remained unchanged. Heart rate was dose dependently increased over control due to reflex tachycardia (Figure 5b). A threshold effect was observed after the 0.1 mg kg^{-1} dose, when heart rate was increased by 17% (52 beats min^{-1}.). A small short lasting elevation of heart rate of about 8% (24 beats min^{-1}) was also observed in all control groups directly after treatment. The maximal increase after application of 0.3, 1.0, 3.0 and 10 mg kg^{-1} was 30% (89), 30% (87), 32% (103) and 58% (164 beats min^{-1}) compared to the initial values, respectively. Before treatment the initial values of heart rate in the treated and control groups varied between 279–321 b.p.m.. For comparison we also administered YC-1 with 100 and 300 mg kg^{-1} orally to conscious SHR. No significant effect on blood pressure and heart rate was observed.

Multiple dosing in conscious SHR

In the single dose study 3 mg kg^{-1} p.o. of BAY 41-8543 reduced blood pressure for more than 24 h. Therefore, we used this dose to investigate the effect of BAY 41-8543 on blood pressure by multiple dosing. The compound was given orally once daily for 4 days. On day 5 placebo and BAY 41-8543 treated animals only received the vehicle (Figure 6a). On days 1–4 MAP maximally decreased by 25, 32, 32, 30%, corresponding to a decrease of 33, 42, 42, 40 mmHg from initial values before the first treatment. The drop of systolic and diastolic blood pressure was comparable (data not shown). In the vehicle treated group blood pressure did not significantly change from the initial conditions. Since the blood pressure lowering effect of 3 mg kg^{-1} p.o. lasted longer than 24 h (Figure 6a); mean arterial blood pressure was lower on days 2 to 4 prior to the next application when compared to the initial value of about 130 mmHg on day 1. It was 115, 117 and 115 mmHg on days 2 to 4. After the last administration of BAY 41-8543 the baseline level was reached about 34 h later. After treatment with BAY 41-8543 heart rate maximally increased from 297 to 450 beats min^{-1} on day 1, from 281 to 386 beats min^{-1} on day 2 and from 296 to 387 beats min^{-1} on day 3 and from 308 to about 405 beats min^{-1} on day 4. In the vehicle treated group the circadian profile of heart rate was not significantly changed (Figure 6b).

Rat tail bleeding time

To determine whether the antiplatelet effects observed *in vitro* (Stasch *et al*., 2002) also occur *in vivo*, we administered a single oral dose of BAY 41-8543 (0.1, 0.3 and 1.0 mg kg^{-1}) to rats and examined the bleeding time prolongation. BAY 41-8543 significantly prolonged rat tail bleeding time from 98 ± 3 s (controls) to 111 ± 8.0 s (0.1 mg kg^{-1} p.o.), to 169 ± 5.0 s (0.3 mg kg^{-1} p.o., $P<0.001$), and to 184 ± 4.0 s (1.0 mg kg^{-1} p.o., $P<0.001$). In this study acetylsalicylic acid (30 mg kg^{-1} p.o.: 221 ± 4.0, $P<0.001$) was used as positive controls.



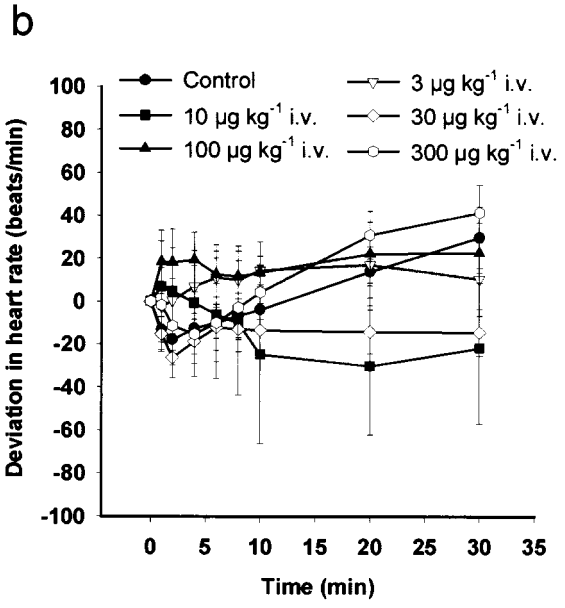


Figure 3 Effect of intravenously administered BAY 41-8543 (0, 3, 10, 30, 100, and 300 μg kg^{-1}) on mean arterial blood pressure (a) and heart rate (b) in anaesthetized rats ($n=4-8$ per group). Values depicted represent changes in mean arterial blood pressure and heart rate. The mean blood pressure at baseline was respectively 105±5, 118±8, 112±7, 115±4, 100±6, and 104±4 mmHg. The mean heart rate at baseline was 329±1, 372±10, 395±23, 336±7, 355±14, and 342±13 beats min^{-1}.

$FeCl_3$ arterial thrombosis model in rats

Administration of 3 mg kg^{-1} p.o. BAY 41-8543 inhibited thrombus formation in the carotid artery by 59±17% as evidenced by the decrease in thrombus mass. In the vehicle treated animals thrombus mass was 2338±218 μg and in the

Table 2 Effects of GTN on MAP (mmHg) during control periods, during infusion of BAY 41-8543 and during simultaneous infusion of BAY 41-8543 and noradrenaline. Values are means ±s.e.mean

	Control (n=5)					*During infusion of BAY 41-8543* (n=5)				
	Control	*GTN 0.5 µg kg^{-1}*	*Control*	*GTN 1 µg kg^{-1}*	*Control*	*GTN 3 µg kg^{-1}*	*BAY 41-8543*	*GTN 0.5 µg kg^{-1}*	*BAY 41-8543*	*GTN 1 µg kg^{-1}*	*BAY 41-8543*	*GTN 3 µg kg^{-1}*
mean	88	72	92	70	86	58	67	56	72	54	64	49
s.e.mean	0.8	3.6	1.9	4.3	1.9	1.6	3.0	3.3	2.4	2.9	2.6	3.4
	Control (n=3)					*during infusion of BAY 41-8543 and noradrenaline* (n=3)						
	Control	*GTN 0.5 µg kg^{-1}*	*Control*	*GTN 1 µg kg^{-1}*	*Control*	*GTN 3 µg kg^{-1}*	*BAY 41-8543*	*GTN 0.5 µg kg^{-1}*	*BAY 41-8543*	*GTN 1 µg kg^{-1}*	*BAY 41-8543*	*GTN 3 µg kg^{-1}*
mean	88	73	89	70	84	59	88	58	89	59	88	51
s.e.mean	3.8	0.9	3.5	1.5	0	1.3	1.5	3.9	2.5	5.2	5	3.8

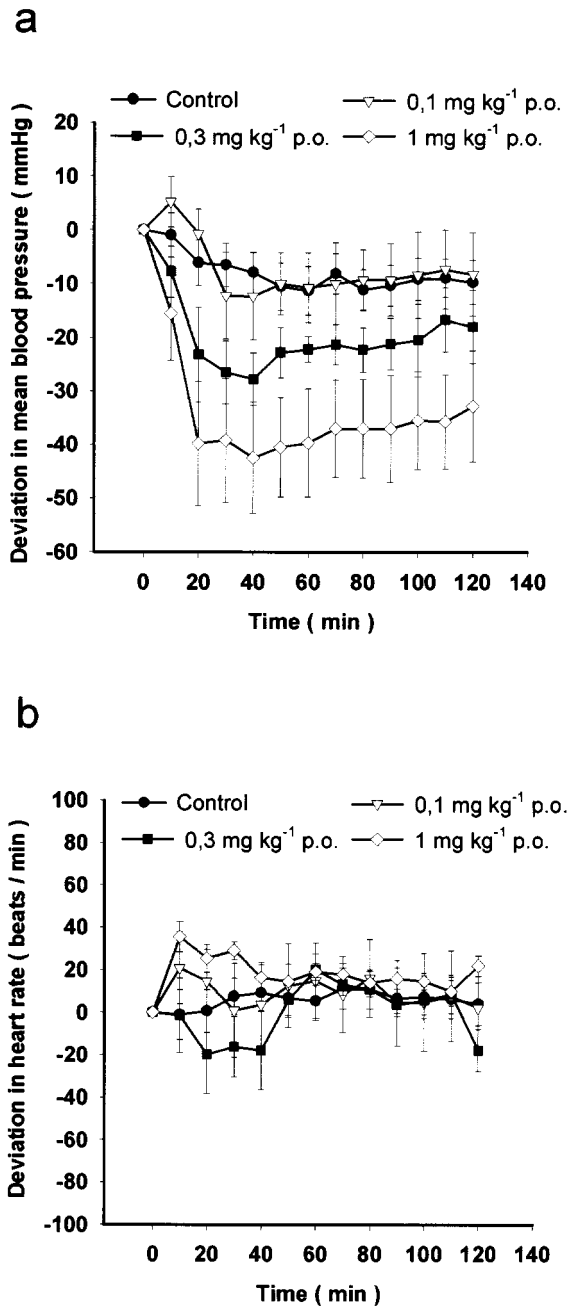


Figure 4 Effect of orally administered BAY 41-8543 (0, 0.1, 0.3 and 1 mg kg^{-1}) on mean arterial blood pressure (a) and heart rate (b) in anaesthetized rats ($n = 4-10$ per group). Values depicted represent changes in mean arterial blood pressure and heart rate. The mean blood pressure at baseline was respectively 125 ± 2, 115 ± 4, 116 ± 5 and 118 ± 7mmHg. The mean heart rate at baseline was 389 ± 9, 369 ± 22, 379 ± 1 and 417 ± 8 beats min^{-1}.

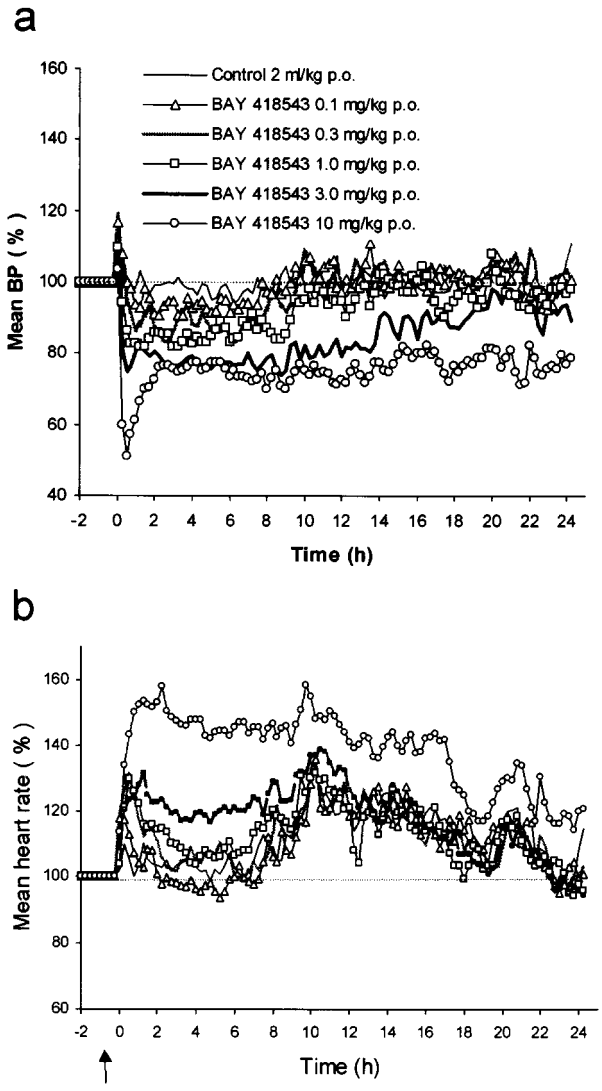


Figure 5 Continuous 24-hour profile of mean blood pressure and heart rate in spontaneously hypertensive rats. Dose response relationship of BAY 41-8543 after single oral treatment (0, 0.1, 0.3, 1, 3 and 10 mg kg^{-1}). Controls were administered vehicle. The animals were treated at 0 h. Given are mean values of six animals as percentage of initial values.

BAY 41-8543 thrombus mass was reduced to 951 ± 152 μg ($P < 0.001$).

Long-term study

The development of the increase in systolic blood pressure in 18-week-old renin transgenic rats (TGR(mRen2)27) on L-NAME treatment could be completely prevented by BAY 41-8543 treatment (3 mg kg^{-1} p.o. twice daily). Systolic blood pressure increased in control animals from 247 ± 3 mmHg to 287 ± 2 mmHg during the observation period of 5 weeks (Figure 7). In BAY 41-8543 treated animals, systolic blood pressure decreased slightly from 248 ± 3 mmHg to 240 ± 2 mmHg during the treatment period of 5 weeks. At the end of the study, the difference in blood pressure reached 47 mmHg. In addition, heart rate was also significantly lower in the treatment group (493 ± 4.7 beats min^{-1} versus 438 ± 3.3 beats min^{-1} $P < 0.001$). BAY 41-8543 dramatically increased survival: during the observation period of 5 weeks, nine out of 14 control animals died, whereas all treated animals survived ($P < 0.05$). Table 3 summarizes the plasma parameters in controls and in BAY 41-8543 treated rats at the end of the study. Although statistical analysis did not show any significant differences, the plasma levels of ANP and its second messenger cyclic GMP tended to be lower in the BAY 41-8543 group than in control animals. Plasma

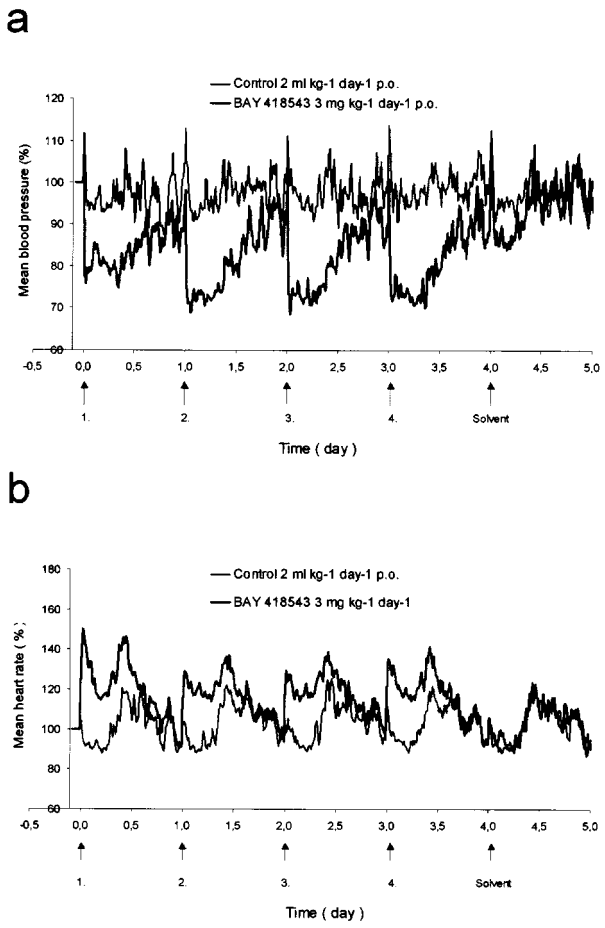


Figure 6 Continuous 24-hour profile of mean blood pressure and heart rate in spontaneously hypertensive rats. Effects of BAY 41-8543 (—) after multiple treatment with 3 mg kg^{-1} p.o.. Controls (—) were administered vehicle. The animals were treated at 0 h. Six animals per group were used.

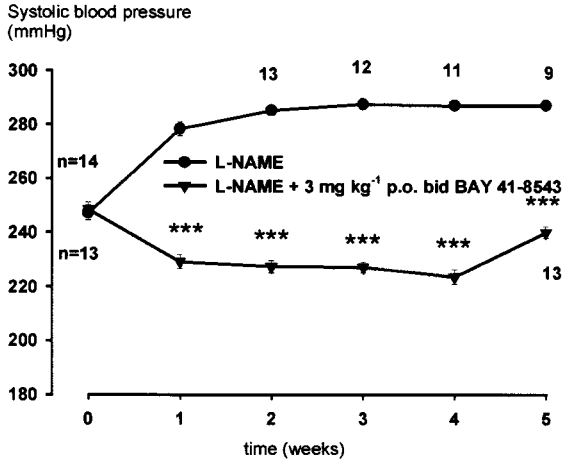


Figure 7 Development of systolic blood pressure in BAY 41-8543-treated (3 mg kg^{-1} p.o. b.i.d.) and untreated TGR(mRen2)27 under L-NAME (500 mg l^{-1} drinking water) for 5 weeks. Numbers over or under the curve show number of surviving rats. Values are means ± s.e.mean. ***$P<0.001$ compared with the values in untreated controls.

creatinine, urea, aldosterone and renin activity were significantly lower in the BAY 41-8543 group. These results show that chronic treatment with the sGC stimulator BAY 41-8543 prevents the increase in blood pressure, shows a kidney protective effect and increased survival in a high renin, low NO rat model of hypertension.

Discussion

Searching for NO-independent sGC stimulators, we selected a series of pyrazolopyridine derivatives that are very potent stimulators of sGC *in vitro* out of around two thousand newly synthesized substances (Straub *et al*., 1998; 2001; Stasch *et al*., 2001). We used the indazole YC-1 (Ko *et al*., 1994; Wu *et al*., 1995) as chemical lead structure and selected BAY 41-8543 as the most potent *in vivo* compound. In the present study we demonstrate that BAY 41-8543 caused a decrease in blood pressure and an increase in coronary blood flow in anaesthetized dogs demonstrating a potent vasodilatory effect *in vivo*. The simultaneously occurring increase in oxygen saturation in the coronary sinus is another indicator of this coronary vasodilation and indicates an oxygen supply to the heart in excess of its demand, i.e. comsumption. This may be beneficial for treatment of patients with coronary artery disease. A similar haemodynamic profile, i.e. lowering of blood pressure and increase in coronary blood flow, is also characteristic for other drugs which are clinically used for treatment of hypertension and/or angina pectoris, e.g. calcium channel blockers and dipyridamol (Akinboboye *et al*., 2001). On theoretical grounds, a steal phenomenon cannot be excluded. However, since no reliable animal models are available to study the steal phenomenon, this question needs to be addressed during the clinical development. On the other hand, an adverse effect of the steal phenomenon on cardiovascular mortality by the treatment with calcium channel blockers has not been substantiated so far.

We also investigated a possible interaction in anaesthetized dogs with respect to the cardiovascular effects, especially the blood pressure lowering effect of BAY 41-8543 and GTN. Administration of GTN during infusion of BAY 41-8543 caused a decrease in mean arterial blood pressure to slightly lower levels when compared to similar applications of GTN in the absence of BAY 41-8543. This effect is not unexpected since two drugs lowering blood pressure by different mechanisms were applied concomitantly and their effects may be additive. However, the decrease in blood pressure by GTN in the presence of BAY 41-8543 was always less than the sum of the decrease in blood pressure by either compound when given alone. This effect may be explained by hormonal or nervous mechanisms preventing a too large decrease in blood pressure in the setting of *in vivo* experiments. Therefore, it is unlikely that during the clinical development of BAY 41-8543 a concomitant application of GTN results in an exaggerated fall in blood pressure. When the decrease in blood pressure by BAY 41-8543 was prevented by concomitant infusion of noradrenaline, GTN evoked a larger decrease in blood pressure compared to its administration in the absence of BAY 41-8543. This may indicate some synergistic effect between NO and BAY 41-8543 as has been described in *in vitro* experiments (Stasch *et al*., 2002).

Table 3 Effects of long-term treatment (5 weeks) with BAY 41-8543 (3 mg kg^{-1} orally twice daily) on plasma renin activity, aldosterone, creatinine, urea, ANP, and cyclic GMP, in transgenic renin rats (TGR(mRen2)27) under L-NAME

	TGR(mRen2)27	
Plasma parameters	*Controls*	*BAY 41-8543*
PRA (ng ml^{-1} h^{-1})	24.1±5.2 (8)	8.5±2.3 (11)**
Aldosterone (pg ml^{-1})	2853±368 (8)	1395±231 (11)***
Creatinine (μmol l^{-1})	107.4±13.6 (9)	68.4±6.5 (11)**
Urea (mmol l^{-1})	21.3±3.3 (9)	10.8±1.2 (11)**
ANP (pg ml^{-1})	1889±780 (8)	1190±355 (10)
cyclic GMP (pmol ml^{-1})	32.6±7.6 (9)	32.6±7.6 (11)

The values are means±s.e.mean (n). $^{**}P<0.01$, $^{***}P<0.001$.

In the anaesthetized dog there is no significant effect of BAY 41-8543 on the venous site and therefore on preload. Another difference between BAY 41-8543 and GTN relates to the effects on coronary vascular resistance. Whereas GTN and other NO donors preferentially dilate large epicardial coronary vessels (Bassenge & Pohl, 1985) without any effect on coronary resistance vessels, BAY 41-8543 caused a profound decrease in coronary resistance. This is evident from the decrease in coronary perfusion pressure and the large increase in coronary blood flow after BAY 41-8543.

NO-independent sGC stimulation by BAY 41-8543 has a strong and dose-dependent blood pressure lowering effect after intravenous administration in normotensive rats with a minimal effective dose of 10 μg kg^{-1} (−16 mmHg) as suggested from the *in vitro* studies (Stasch *et al*., 2002). To achieve a comparable blood pressure lowering effect in rats after intravenous administration, a 500-fold higher dose of YC-1 is needed as a consequence of its weak vasodilatory activity (Mülsch *et al*., 1997; Rothermund *et al*., 2000; Straub *et al*., 2001a). In addition, the blood pressure reduction after 5 mg kg^{-1} i.v. YC-1 lasted for only 6 min in the anaesthetized rat (Rothermund *et al*., 2000), whereas the blood pressure lowering effect of intravenous administered BAY 41-8543 was still present after 2 h at the end of the study. As presented in this study, YC-1 is devoid of any relevant antihypertensive activity in conscious SHR after oral administration. Therefore, one of our aims in the structural optimization process was the development of compounds with a potent and long-lasting blood pressure lowering effect after oral administration. In the anaesthetized normotensive rat, BAY 41-8543 reduces blood pressure with a minimal effective dose of 0.3 mg kg^{-1} p.o. BAY 41-8543 is 3 fold more potent than BAY 41-2272 (Straub *et al*., 2001a). Moreover, BAY 41-8543 showed a dose-related and long-lasting antihypertensive effect in conscious SHR with a minimal effective dose of 0.1–0.3 mg kg^{-1} p.o.. The observed dose-dependent increase in heart rate is due to reflex tachycardia. After oral administration of 3 mg kg^{-1} a 24-hour blood pressure lowering effect was observed. Also in the conscious SHR, BAY 41-8543 is about 3 fold more potent than BAY 41-2272 (Stasch *et al*., 2001). In addition, very recently it has been shown that BAY 41-8543 and BAY 41-2272 *in vivo* form different metabolites by oxidation that display strong sGC stimulating and vasodilatory properties and thereby contribute to the observed haemodynamic effects (Straub *et al*., 2001b).

Despite the widespread use of organic nitrates in the treatment of angina, the development of tolerance limits the therapeutic value of this class of compounds for chronic treatment (Feelisch, 1998). This is believed to be the result, at least in part, of decreased metabolic activation of the compound or excessive superoxide, endothelin or angiotensin II levels (Münzel *et al*., 1996; Parker, 1989). Therefore, compounds which can stimulate sGC in a NO-independent manner may show a significant advantage over existing nitrovasodilator therapy. NO-independent sGC stimulators may activate sGC to cause the same beneficial outcome as organic nitrates yet not suffer from tolerance following repeated administration (Hobbs, 2000). Alternatively, following the use of organic nitrates and the onset of tolerance, sGC stimulators are still able to activate sGC potently as shown in the *ex* vivo studies with BAY 41-8543 (Stasch *et al*., 2002). A 3-day treatment of organic nitrates leads to a strong induction of nitrate tolerance in rats (Mülsch *et al*., 2001). Therefore, we studied the blood pressure lowering effects of BAY 41-8543 after multiple administration over 4 days to conscious spontaneously hypertensive rats. After each dosage the blood pressure had still not fully recovered to basal values after 24 h, i.e. at the time of the next administration. The maximal blood pressure lowering effect was nearly the same after each administration. Therefore, we conclude that BAY 41-8543 does not develop tolerance in conscious rats as NO donors, i.e. after a few days.

The significance of the anti-platelet effects *in vitro* (Stasch *et al*., 2002) was confirmed by the *in vivo* results. We observed a significant prolongation in rat tail bleeding time. In this *in vivo* model a more than 300-fold higher dose of YC-1 is needed to show an effect comparable to BAY 41-8543 (Becker *et al*., 2000). More importantly BAY 41-8543 reduces potently thrombus formation in the $FeCl_3$ thrombosis rat model. The increase in platelet cyclic GMP, the phosphorylation of VASP, the anti-platelet effects *in vitro* (Stasch *et al*., 2002), the anti-thrombotic effect and the prolongation of rat tail bleeding time not only support the role of VASP as an important link between cyclic GMP signal transduction pathway and platelet aggregation but also demonstrate the potential of BAY 41-8543 for the development of a new class of anti-aggregatory and anti-hypertensive drugs.

NO is synthetized in endothelial cells from L-arginine by NO synthase, which can be inhibited by L-arginine analogues such as L-NAME. Both acute and chronic inhibition of NO synthase induce an increase in blood pressure in different rat strains and other experimental animals (Morton *et al*., 1993; Navarro *et al*., 1994; Zatz & Baylis, 1998; Vallance *et al*., 1998). The cardiovascular consequences of sGC stimulation were evaluated by determining the compound's long-term effects on haemodynamic and hormonal parameters in a high renin, low NO rat model of hypertension to show whether this compound is still active *in vivo* under reduced NO levels. In this study we used transgenic rats with an additional renin gene (TGR(mRen2)27) which represent a very sensitive model for the cardiovascular effects of compounds interacting with the NO/sGC system (Hirth-Dietrich *et al*., 1994; Gardiner *et al*., 1998). Systolic blood pressure increased in old renin transgenic rats (TGR(mRen2)27) receiving the NO synthase inhibitor L-NAME in the drinking water, whereas it decreased slightly during the observation period of 5 weeks in animals treated with both L-NAME and the sGC

stimulator BAY 41-8543. In addition, the heart rate was significantly lower in the treatment group. A 3 fold lower dosage of BAY 41-8543 is needed to show similar haemodynamic effects like BAY 41-2272 in this experimental model (Stasch *et al*., 2001). At the end of the study, renin activity, aldosterone, urea and creatinine in plasma had significantly decreased reflecting a kidney protective effect of BAY 41-8543. Moreover, these results clearly show that BAY 41-8543 acts under *in vivo* conditions of low endogenous NO. The beneficial effects of BAY 41-8543 in this therapeutically relevant animal model of hypertension are also emphasized by a significant reduction in mortality without any signs of development of tolerance.

Due to its ubiquitous nature, the pathogenesis of various disease states has been linked to aberrations in sGC signalling. This is especially true of the cardiovascular system, where inappropriate activation of sGC may underlie conditions such as stroke, atherosclerosis, erectile dysfunction, and septic shock; however, altered sGC activation has also been associated with the pathogenesis of asthma and certain neurodegenerative disorders (Hobbs, 2000).

Taken together, our findings demonstrate that NO-independent stimulation of sGC by BAY 41-8543 is a powerful blood pressure lowering agent after intravenous and oral administration. BAY 41-8543 increases coronary blood flow and oxygen saturation in the coronary sinus blood. In addition, an anti-thrombotic effect and a prolongation of bleeding time were observed. The overall beneficial effects of BAY 41-8543 manifested as both anti-platelet effect and vasodilatation were shown in a long-term study in a low NO and high renin model of hypertension without any signs of tolerance. The cardiovascular profile of BAY 41-8543 suggests therefore a unique clinical utility for the treatment of cardiovascular diseases.

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