Full Paper

Arylazoamidoximes and Related Compounds as NO-modulators

Alexander Schröder[1], Jürke Kotthaus[2], Dennis Schade[2], Bernd Clement[2], and Klaus Rehse[1]

[1] Pharmaceutical Institute, Freie Universität Berlin, Berlin, Germany
[2] Pharmaceutical Institute, Christian-Albrechts-University of Kiel, Kiel, Germany

Three amidinoarylhydrazines **1**, three arylazoamidines **2**, and nine arylazoamidoximes **3** have been synthesized and investigated for their potential to function as nitric oxide (NO) modulators. *In-vitro* studies demonstrated that **2** and **3** inhibited platelet aggregation (**2c**, IC_{50} = 3 μM) which could also be shown *in vivo* by inhibition of thrombus formation in arterioles (**3a**, 22%). Moreover, for all compounds antihypertensive effects were examined *in vivo* with SHR rats, with **2a** being the most potent candidate by lowering blood pressure by 19%. However, no common underlying mechanism of action could be shown. Some of these compounds released HNO non-enzymatically. Incubations with NO synthase isoforms (NOSs) revealed, that compounds **1** to **3** were weak substrates for NOSs but arylazoamidoximes **3** remarkably elevated the NOSs activity in the presence of L-arginine (**3h**, up to fivefold). In addition, we examined effects on arginase and dimethylarginine dimethylaminohydrolase (DDAH), two further enzymes involved in the complex regulation of NO biosynthesis, to elucidate whether the observed *in-vivo* effects can be traced back to their modulation. Furthermore, the metabolic fate of arylazoamidoximes **3** was addressed by investigation of a possible *N*-reductive biotransformation. In summary, novel NO-modulating compound classes are presented, among which arylazoamidoximes **3** are potent activators of NOS isoforms, and arylazoamidines **2** exert *in-vivo* effects by unknown mechanisms.

Keywords: Antihypertensive / Antiplatelet / Antithrombotic / HNO – NO formation / NOS activation

Received: March 20, 2009; Accepted: July 7, 2009

DOI 10.1002/ardp.200900060

Introduction

Nitric oxide (NO) is endogenously formed from L-arginine via nitric oxide synthases (NOSs) and is involved in various physiological processes, such as regulation of vascular tone, inhibition of platelet aggregation, and leukocyte adhesion on the endothelial surface [1]. In addition, it is generated in large quantities by macrophages during host defence and immunological reactions as well as by neurons of the central nervous system, where it acts as a neuromediator in basic physiological functions, including modulation of pain [1, 2]. Accordingly, it is vital that NO formation is strictly regulated, and it is not surprising that dysregulation leads or contributes to the development of numerous diseases [3]. Insufficient NO availability is associated with hypertension [4], coronary heart disease [5], heart failure and myocardial infarction as well as erectile dysfunction [6]. The predominant enzymes in this regulation process are NOSs, arginases, and dimethylarginine dimethylaminohydrolases (DDAHs).

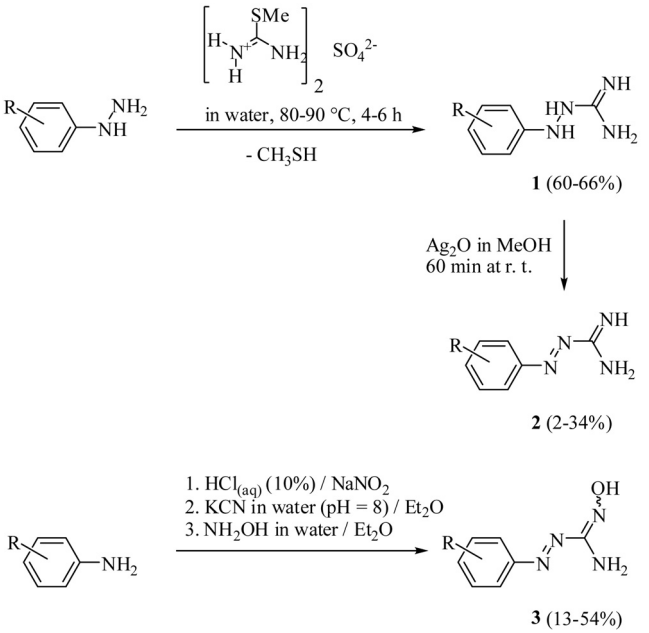
Physiologically, NOSs activity is regulated by the endogenous, competitive inhibitors asymmetric N^{ω},N^{ω}-dimethyl-L-arginine (ADMA) and N^{ω}-monomethyl-L-arginine (NMMA). These compounds, which are released by proteolysis of various proteins containing methylated arginine residues, are degraded by DDAHs to L-citrulline and either dimethylamine or methylamine [7].

Correspondence: Prof. Dr. Klaus Rehse, Pharmaceutical Institute – Freie Universität Berlin, Königin-Luise-Str. 2 + 4, D-14195 Berlin, Germany.
E-mail: rehiwer@zedat.fu-berlin.de
Fax: +49 30 304-2925

Abbreviations: dimethylarginine dimethylaminohydrolases (DDAHs); nitric oxide (NO); nitric oxide synthases (NOSs); spontaneously hypertensive rat (SHR)

Interestingly, a regulatory mechanism between arginase and NOSs becomes more and more apparent, since these metabolic pathways compete for their common substrate L-arginine [8]. Essentially, arginase inhibition leads to increased NO levels by elevating the substrate pool for NOSs.

In this paper, we have concentrated on amidinoarylhydrazines **1**, arylazoamidines **2**, and arylazoamidoximes **3**, which were expected to be alternate substrates for NOSs or, respectively, would release NO by other mechanisms. Therefore, typical NO-dependent effects were investigated by several methods such as the inhibition of platelet aggregation, antithrombotic effects or by blood pressure measurements in SH (spontaneously hypertensive) rats. Moreover, in this study we examined if the observed effects depend on NOS activation or were due to a modulation of arginase or DDAH, two other enzymes of the NO generating system.



Scheme 1. Synthesis of amidinoarylhydrazines **1**, arylazoamidines **2**, and arylazoamidoximes **3**. For details see Experimental.

Results and discussion

Chemistry

The synthesis of type **1**–**3** compounds was performed according to modified literature procedures (see Scheme 1). Reaction of hydrazines with *S*-methylisothiouronium sulfate yield amidinoarylhydrazines **1** in 60–66% overall yield [9]. Oxidation with silver oxide delivered arylazoamidines **2** in only hardly reproducible manner [10]. Diazotation of anilines and reaction with cyanide yield arylazonitriles which were not isolated but converted further to the arylazoamidoximes **3** with hydroxylamine [11].

Biology

Secondary effects of nitric oxide formation were measured by the following methods. Two *in-vivo* test systems were used to examine the relevance of the *in-vitro* results for therapeutical purposes.

In-vitro effects on platelet aggregation

First, stimulation of guanylate cyclase by nitric oxide and subsequent inhibition of blood platelet aggregation was determined via the Born test [12]. As can be seen in Table 1, two classes of compounds (**2**, **3**) inhibited platelet aggregation. Arylazoamidines **2** represent the most potent compounds with IC_{50} values between 3 to 30 μM. Moreover, the results showed that a 4-fluoro-substitution of **2c** is superior to a 4-Cl moiety. In addition, by comparing **2a** and **2b** a substitution in position 4 seems to improve the potency. This is also underlined by the results of type **3** compounds. Within these compounds **3a**, **3c**, and **3f** were almost as effective as **2b**, whereas all other substitution patterns were less favorable.

In-vivo antithrombotic effects

In the laser thrombosis assay format, compound **3a** showed the best potency (see Table 1). Arylazoamidines **2**, which seemed to be superior in the Born test, were less effective in this *in-vivo* study possibly due to low absorption after oral application. At physiological pH, these compounds are positively charged which impairs absorption. Amidoximes are much less basic and therefore show improved oral bioavailability [13].

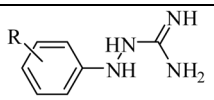
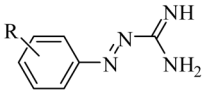
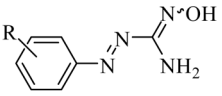
In-vivo effects on blood pressure

The blood pressure measurement showed that most compounds reduced blood pressure in SHR rats, among which the most active candidates were **1a**, **1b**, **2a**, **2c**, **3a**, **3c**, and **3d** (see Table 1). Obviously, 4-substituted substances of all classes (**1**–**3**) were particularly potent. However, the observed antihypertensive potency of arylazoamidines **2** appeared surprising, because of their lack of potency in the laser-thrombosis model which can be explained by a poor oral bioavailability (see above).

In-vitro NO formation by NOS isoforms

As compounds **1**–**3** are structurally similar to *N*-hydroxyguanidines, they were thus examined as alternate substrates for NOSs or, respectively, as activators of the enzyme (see Table 2). NO formation was measured indirectly by detecting nitrite via the Griess assay.

Table 1. Results of compounds **1a–c**, **2a–c** and **3a–i** in the Born-, laser thrombosis and blood pressure test.

Compound	R	Born-test[a] IC_{50} (μM)	Laser thrombosis test[b] Inhibition in arterioles (%) 60 mg/kg	Blood pressure test[c] Max. Blood-pressure reduction after 2 h (%) 30 mg/kg	60 mg/kg
1-Amidino-2-arylhydrazines					
1a	4-Cl	>300	7 ± 1	17 ± 4	n. m.
1b	3-Cl	>300	7 ± 2	7 ± 6	n. m.
1c	4-F	>300	6 ± 2	9 ± 5	n. m.
Arylazoamidines					
2a	4-Cl	14	8 ± 1	13 ± 7	19 ± 4
2b	3-Cl	30	4 ± 1	n. s.	n. m.
2c	4-F	3	n. s.	11 ± 5	n. m.
Arylazoamidoximes					
3a[d]	4-Cl	37	22 ± 2	10 ± 3	10 ± 3
3b	3-Cl	160	3 ± 2	n. s.	4 ± 3
3c[d]	4-F	25	n. s.	8 ± 3	12 ± 2
3d	2-Cl	210	7 ± 1	n. m.	14 ± 6
3e	3-F	170	9 ± 2	n. m.	15 ± 14
3f	2-F	30	10 ± 2	n. m.	5 ± 3
3g	4-CF_3	230	6 ± 1	n. m.	6 ± 5
3h	4-phenyl	210	9 ± 2	n. m.	n. s.
3i	2,6-Cl	150	7 ± 2	n. m.	n. s.

[a] Born-test: *In-vitro* inhibition of platelet aggregation induced with collagen.
[b] laser thrombosis test: *In-vivo* inhibition of laser-induced thrombosis formation in mesenterial vessels of male Wistar rats after a single oral administration.
[c] blood-pressure test: Blood-pressure reduction of hypertensive rats, measured at the rat tail after a single oral administration.
[d] described by Bade [30]; n. m. = not measured; n. s. = not significant.

Most arylazoamidoximes **3** enhanced nitrite formation in the presence of L-arginine, whereas in the absence of L-arginine only small amounts of nitrite were detectable. The biphenyl derivative **3h** showed a 34% release of nitrite compared to L-arginine by nNOS. However, in the presence of L-arginine the activation of NOSs was much greater. In particular, this is striking for **3h** which activates nNOS threefold, eNOS fourfold, and iNOS even fivefold. In addition, **3a** and **3i** were also potent activators of NOS isoforms. In summary, all type-**3** compounds represent weak substrates for NOSs but potentiate NOSs activity substantially in the presence of L-arginine, however, it has to be noted that the employed Griess-assay format is susceptible to artifacts, which we cannot entirely rule out. Though, this study may at least provide a clue for the underlying mechanism for the observed *in-vivo* effects. Further studies will be performed to investigate the activation of NOS isoforms more precisely.

It is remarkable that **3h** is the most potent activator of NOSs *in vitro*, since it showed low activity in *in-vivo* models. This might be explained by a possible *N*-reductive biotransformation to the respective arylazoamidine which is well recognized for *N*-hydroxyguanidines and amidoximes [13, 14]. Ambiguously, all other arylazoamidoximes **3** reveal *in-vivo* efficacies but should be metabolically reduced as well.

Non-enzymatical release of HNO and NO

In order to examine whether the observed *in-vivo* effects are due to a non-enzymatical release of NO or HNO – both of them are attributed several biological effects [15] – compounds **1–3** were incubated for 1 h under anaerobic condi-

Table 2. Incubations of compounds **1**–**3** (1 mM) with NO-synthase isoenzymes (nNOS, eNOS, iNOS) in the absence and presence of L-arginine (0.5 mM).[a)]

Compound	R	nNOS	eNOS	iNOS	nNOS + L-arg.	eNOS + L-arg.	iNOS + L-arg.
1-Amidio-2-arylhydrazines							
1a	4-Cl	n. s.	n. s.	n. s.	69 ± 20	22 ± 3	46 ± 4
1b	3-Cl	n. s.	n. s.	n. s.	n. m.	n. m.	n. m.
1c	4-F	n. s.	n. s.	n. s.	34 ± 3	13 ± 2	12 ± 1
Arylazoamidines							
2a	4-Cl	n. s.	18 ± 2	19 ± 8	67 ± 13	29 ± 2	67 ± 7
2b	3-Cl	n. s.	n. s.	n. s.	n. m.	n. m.	n. m.
2c	4-F	n. s.	27 ± 3	11 ± 3	25 ± 1	9 ± 5	31 ± 3
Arylazoamidoximes							
3a	4-Cl	n. s.	n. s.	n. s.	332 ± 9	388 ± 25	484 ± 29
3b	3-Cl	n. s.	n. s.	n. s.	103 ± 3	107 ± 2	112 ± 7
3c	4-F	n. s.	10 ± 1	28 ± 11	140 ± 12	105 ± 10	149 ± 2
3d	2-Cl	n. s.	17 ± 2	17 ± 7	107 ± 2	115 ± 3	113 ± 8
3e	3-F	n. s.	n. s.	n. s.	100 ± 4	99 ± 4	100 ± 8
3f	2-F	n. s.	11 ± 7	12 ± 9	93 ± 3	96 ± 6	87 ± 13
3g	4-CF_3	16 ± 3	9 ± 2	8 ± 6	n. m.	n. m.	n. m.
3h	4-phenyl	34 ± 3	15 ± 2	20 ± 13	310 ± 7	387 ± 10	507 ± 5
3i	2,6-Cl	n. s.	4 ± 3	n. s.	183 ± 5	150 ± 30	193 ±15

[a)] Nitrite formation (%) compared to a control with 0.5 mM L-arginine as substrate (100%); n. m. = not measured; n. s. = not significant.

tions in phosphate buffer in the presence of trifluoroacetoxy-iodosobenzene as an oxygen-donor at 37°C. Furthermore, to mimic a CYP450-mediated HNO- or NO-release, a synthetic Fe^{III}-porphyrine complex (Fe^{III}TPPCl) was added to the incubation mixture [16, 17]. HNO was indirectly detected by measuring its stable decomposition product N_2O by gas chromatography [18], whereas NO could be detected by chemiluminescence [19].

Only for the arylazoamidoximes **3** N_2O could be detected (**3a**: 5.7%, **3c**: 4.5%) as a result of simple chemical degradation. In the presence of Fe^{III}TPPCl the formation of N_2O was significantly enhanced (**3a**: 14.9%, **3c**: 19.3%, **3g**: 4.3%). These findings indicate possible mechanisms for a purely chemically and CYP450-mediated release of HNO *in vivo* which may be responsible for the observed biological effects. In contrast, the formation of NO was not significant for all compounds.

Effects on bovine arginase and human DDAH-1

Exemplary for all arylazoamidines and arylazoamidoximes, the effects of **2a**, **3a**, and **3c** on bovine liver arginase and recombinantly expressed human DDAH-1 were examined. These enzymes are involved in the complex regulation of NO bioavailability and might be responsible for the effects observed in *in-vivo* studies. An additional inhibition of arginase would enhance NO formation, whereas inhibition of hDDAH-1 would impair NO biosynthesis.

Our studies clearly showed that these compounds do not affect activity of arginase and DDAH-1 at concentrations of 100 and 500 μM. Thus, the observed *in-vivo* effects seem to be due to an increase in NOSs activity. Particularly **2a** was expected to affect one of these enzymes, since it showed significant antihypertensive effects and no modulation of NO, neither direct via NOSs nor non-enzymatically. This phenomenon could have been explained by an arginase inhibition. Obviously, this compound reduces blood pressure by a different mode of action. A possible explanation would be that arylazoamidines **2** have structural similarities to guanfacine, which lowers blood pressure by activating a_2-receptors [20].

Moreover, our investigations revealed that these compounds are not converted as alternate substrates, neither by arginase nor DDAH-1.

Reduction of arylazoamidoximes

Meanwhile, a reduction of *N*-hydroxylated guanidines and amidines is well known [13, 14]. Therefore, we examined a possible physiological reduction of type-**3** compounds. **3a**, **3b**, and **3c** were reduced by human and porcine mitochondria, and microsomes purified from liver and kidney. **3a** is most intensely reduced with a maximal reduction rate of 17.16 nmol/(min mg) by human liver microsomes. Reduction rates for **3b** and **3c** are lower (for detailed reduction rates see Table 3). This might, in part, explain the differences in the described *in-vitro* and *in-*

Table 3. Reduction rates of arylazoamidoximes **3** to the corresponding arylazoamidines **2** by different enzyme sources.

	Specific activity (nmol/(min mg protein)		
	3a 4-Cl	**3b** 3-Cl	**3c** 4-F
Mitochondria			
Human liver	1.22	0.10	n. s.
Human kidney	3.25	0.12	n. s.
Porcine liver	0.81	0.17	1.04
Porcine kidney	3.92	0.17	1.77
Microsomes			
Human liver	17.16	0.38	2.89
Human kidney	6.17	0.12	n. s.
Porcine liver	1.68	0.11	0.11
Porcine kidney	1.99	0.12	0.20

n. s. = not significant

vivo studies. In contrast to arylazoamidines **2**, the investigated arylazoamidoximes **3** were potent activators of all NOS isoforms *in vitro*, whereas their *in-vivo* effects on blood pressure and thrombosis formation were comparable to the arylazoamidines. Probably, arylazoamidoximes are physiologically reduced which seems to limit their effects *in vivo*.

Conclusions

Within our investigations, arylazoamidines **2a**, **2c** and arylazoamidoximes **3a**, **3c** turned out to be the most potent compounds. Thus, chloro- or fluoro-substitution, respectively, in position 4, seemed to be most favorable. These compounds inhibited platelet aggregation and thrombosis formation and also lowered blood pressure of SHR rats. We assume that the observed effects were due to a release of NO either by the compounds themselves or by an increase in NOSs activity. Our studies clearly revealed that they did not liberate NO non-enzymatically, but to a small extent the release of HNO was observed, which might, in part, explain their biological effects.

Moreover, our studies with NOS isoforms showed that arylazoamidines **2** and arylazoamidoximes **3** were only weak alternate substrates for NOSs. However, **3a**, **3c**, and **3h** were capable of enhancing NOSs activity in the presence of L-arginine up to fivefold. Despite this considerable activation of NOSs, the *in-vivo* effects on blood pressure were comparable to that of **2a** and **2c**, which seem to be due to a rapid physiological *N*-reduction to the corresponding arylazoamidines. In addition, it is striking that **2** did not enhance NOSs activity, whereas these compounds effectively lowered blood pressure *in vivo*. This might be caused by a second mode of action such as the stimulation of a_2-receptors, considering their structural similarity to guanfacine. Any further possible modulation of the NO generating system explaining these effects was ruled out by investigations with arginase and DDAH.

Experimental

Chemistry

M.p. (uncorr.): Linström apparatus (Bühler, Tübingen, Germany). Elemental analysis: Elementar Vario EL (Elementar, Germany). IR: Perkin-Elmer 1420 Ratio Recording IR-Spectrophotometer (Perkin-Elmer, Norwalk, CT, USA). ATI Mattson Genesis Serie FTIR (Unicam Analytische Systeme, Kassel, Germany). NMR: Bruker AC 300 and Bruker Avance / DPX 400 (Bruker Bioscience, USA). EI-MS: CH-7A-Varian MAT (70 eV; Varian, USA) and Kratos MS 25 RF (80 eV; Kratos Analytical, UK).

The synthesis of amidinoarylhydrazines **1a** and **1c** has already been reported [21]. **3a** and **3c** were synthesized as described by Rehse *et al.* [22]. All other compounds were prepared for the first time.

Wistar rats for the *in-vivo* tests were purchased at Tierzucht Schönwalde, GmbH.

*General procedure for the synthesis of 1-amidino-2-arylhydrazines **1***

According to a modified method of Ohuchida *et al*. [9], 26 mmol of the aromatic hydrazine · HCl are mixed with equal molaric amounts of NaOH and *S*-methylisothiourea-sulfate in an aqueous solution at 80–90°C and stirred for 4–6 h. After cooling and removing of solvent the residue is boiled in absolute ethanol and filtered hot. The filtrate is concentrated in a vacuum and the crude product recrystallized.

*1-Amidino-2-(4-chlorophenyl)-hydrazine-monohydrate **1a** [9]*

From 3,29 g 4-chlorophenylhydrazine · HCl (18,6 mmol) and 2 g (10,7 mmol) *S*-methylisothioureasulfate. Beige crystals (H_2O), m.p.: 213°C, yield: 1.3 g (66%). Anal. $C_7H_{11}ClN_4O$ (202). IR (KBr) ν [cm^{-1}]: 3465, 3357, 3199, 3118, 3001, 2857, 1881, 1674, 1647, 1589, 1492, 1435, 1260, 1088, 825; ^{1}H-NMR (400 MHz, DMSO-d_6) δ [ppm]: 6.76 (d, *J* = 8.7 Hz, 2H, aromat. 2-H, 6-H), 7.23 (d, *J* = 8.7 Hz, 2H, aromat. 3-H, 5-H), 7.44 (br. s, 1H, D_2O exchange, ClC_6H_4NHNH), 7.69 (br. s, 1H, D_2O exchange, ClC_6H_4NH), 8.23 (s, 2H, D_2O exchange, $-NH_2$), 9.61 (s, 1H, D_2O exchange, =NH); MS (EI, 210°C) *m*/*z* (%): 184 (86) [$M^{+\bullet}$], 167 (44), 142 (18), 132 (27), 125 (100), 111 (21), 99 (18), 90 (25), 43 (37), 36 (53), 28 (11).

*1-Amidino-2-(3-chlorophenyl)-hydrazine-monohydrate **1b***

From 3.29 g 3-chlorophenylhydrazine · HCl (18.6 mmol) and 2 g (10.7 mmol) *S*-methylisothioureasulfate. Beige crystals (ethylacetate), m.p.: 164°C, yield: 1.2 g (61%). Anal. $C_7H_9ClN_4$ (202). IR (KBr) ν [cm^{-1}]: 3435, 3215, 3156, 3054, 1672, 1644, 1598, 1502, 1476, 1415, 685; ^{1}H-NMR (400 MHz, DMSO-d_6) δ [ppm]: 6.75 (m, 2H, aromat. 2-H, 6-H), 6.89 (d, *J* = 8.1 Hz, 1H, aromat. 4-H), 7.27 (t, 1H, aromat. 5-H), 8.13 (s, 2H, D_2O exchange, NH_2), 8.43 (s, 1H, D_2O

exchange, =NH), 10.01 (s, 2H, D_2O exchange, NH-NH); MS (EI, 330°C) *m/z* (%): 184 (100) [$M^{+\bullet}$], 167 (46), 142 (23), 132 (57), 125 (69), 111 (23), 90 (30), 75 (16), 43 (72), 36 (80).

1-Amidino-2-(4-fluorophenyl)-hydrazine-dihydrate ***1c*** *[9]*

From 4.9 g 4-fluorophenylhydrazine · HCl (30 mmol) and 4.2 g (30 mmol) *S*-methylisothioureasulfate. Beige crystals (THF), m.p.: 165°C, yield: 3.0 g (60%). Anal. $C_7H_{13}FN_4O_2$ (204.2). IR (KBr) ν [cm^{-1}]: 3443, 3380, 3290, 3200, 3136, 3009, 1668, 1644, 1511, 1212, 829, 631. ^{1}H-NMR (400 MHz, DMSO-d_6) δ [ppm]: 6.75 (dd, *J* = 9.0 Hz, *J* = 6.8 Hz, 2H, aromat. 2-H, 6-H), 7.08 (t, *J* = 8.8 Hz, 2H, aromat. 3-H, 5-H), 7.44 (br. s, 1H, D_2O exchange, FC_6H_4NHNH), 7.67 (br. s, 1H, D_2O exchange, FC_6H_4NHNH), 8.11 (s, 2H, D_2O exchange, $-NH_2$), 9.64 (s, 1H, D_2O exchange, =NH); MS (EI, 80°C) *m/z* (%): 168 (?) [$M^{+\bullet}$], 126 (100), 110 (71), 95 (17), 83 (74), 57 (39), 43 (33), 36 (82), 28 (51).

General procedure for the synthesis of arylazoamidines ***2***

According to a modified method of Henrion [10], 10 mmol of the respective 1-amino-2-arylhydrazine **1** are dissolved in 20 mL of methanol and suspended with 20 mmol of freshly precipitated silver oxide for 60 min at room temperature. After filtration arylazoamidines **2** are precipitated from the filtrate as their hydrochloride or maleate.

4-Chlorophenylazoamidine · HCl ***2a***

From 1.24 g **1a** (6.7 mmol) and 3.11 g (13.44 mmol) silver oxide. Red crystals, m.p.: 152°C, yield: 0.5 g (34%). Anal. $C_7H_8Cl_2N_4$ (219.1). IR (KBr) ν [cm^{-1}]: 3282, 3142, 3089, 1720, 1485, 1447, 1407, 1281, 1165, 1153, 1088, 1014, 922. ^{1}H-NMR (400 MHz, DMSO-d_6) δ [ppm]: 7.81 (d, *J* = 8.7 Hz, 2H, aromat. 3-H, 5-H), 8.00 (d, *J* = 8.7 Hz, 2H, aromat. 2-H, 6-H), 9.65 (s, 2H, D_2O exchange, NH_2), 9.78 (s. 2H, D_2O exchange, NH_2^+); MS (EI, 50°C) *m/z* (%): 183 (0) [$M^{+\bullet}$], 111 (11), 43 (100).

3-Chlorophenylazoamidine-maleate ***2b***

From 3.0 g **1b** (16.2 mmol) and 8.0 g (34.5 mmol) silver oxide. Beige powder, m.p.: 131°C, yield: 0.1 g (2%). Anal. $C_{11}H_{13}ClN_4O_4$ (300.7). IR (KBr) ν [cm^{-1}]: 3343, 3072, 1731, 1623, 1573, 1475, 1388, 1361, 1195, 1071, 863; ^{1}H-NMR (400 MHz, DMSO-d_6) δ [ppm]: 6.16 (s, 2H, maleat.), 7.85 (t, *J* = 8.0 Hz, 1H, aromat. 5-H), 7.97 (d, 1H aromat. 4-H), 8.02 (s, 1H, aromat. 2-H), 8.08 (d, *J* = 8.0 Hz, 1H, aromat. 6-H), 9,78 (m, 4H, D_2O exchange, NH_2, NH_2^+); MS (EI, 90°C) *m/z* (%): 184 (2) [$M^{+\bullet}$], 111 (13), 99 (14), 75 (10), 72 (96), 43 (100), 55 (31), 27 (49).

4-Fluorophenylazoamidin-maleate ***2c***

From 2.97 g **1c** (17.7 mmol) and 6.0 g (26.0 mmol) silver oxide. Yellow powder, m.p.: 159°C, yield: 0.5 g (6%). Anal. $C_{11}H_{11}FN_4O_4$ (282.2). IR (KBr) ν [cm^{-1}]: 3433, 1720, 1630, 1504, 1367, 1237, 1141, 852, 794; ^{1}H-NMR (400 MHz, DMSO-d_6) δ [ppm]: 5.95 (s, 2H, maleinic acid), 7.53 (t, *J* = 8.6 Hz, 2H, aromat. 3-H, 5-H), 8.01 (dd, *J* = 8.5 Hz, *J* = 5,2 Hz, 2H, aromat. 2-H, 6-H), 9.62 (m, 4H, D_2O exchange, NH_2, NH_2^+), MS (EI, 90°C) *m/z* (%): 166 (?) [$M^{+\bullet}$], 123 (1), 95 (10), 72 (13), 43 (100).

General procedure for the synthesis of arylazoamidoximes ***3***

According to Longo [11], 40 mmol of the respective aromatic amine was dissolved or suspended in 50 mL of 10% HCl and oxidized with $NaNO_2$ below 5°C. The mixture was adjusted to pH = 8 with Na_2CO_3. Diethylether was added and the two-phase system was stirred with 36 mmol KCN until the color difference of the two phases was stable (ca. 30 min). The ether phase was collected and stirred with an aqueous solution of 36 mmol hydroxylamine · HCl and 63 mmol NaOH until the color difference of the two phases was stable (20 to 60 min). The aqueous phase was washed with diethylether and, thereafter, acidified with glacial acetic acid. The precipitate was recrystallized.

3-Chlorophenylazoamidoxime-semihydrate ***3b***

From 5.1 g 3-chloraniline (40 mmol) and 2.50 g (36 mmol) hydroxylamine · HCl. Crystals (ethylacetate/*n*-hexane), m.p.: 162°C, yield: 2,3 g (29%). Anal. $C_7H_7ClN_4O \cdot 1/2\ H_2O$ (207.61). IR (KBr) ν [cm^{-1}]: 3481, 3359, 3073, 2819, 1681, 1552, 1475, 1451, 1414, 1020, 979, 780; ^{1}H-NMR (400 MHz, DMSO-d_6) δ [ppm]: 5.91 (s, 2H, NH_2, D_2O exchange), 7.62 (dd, *J* = 4.3 Hz, *J* = 3.3 Hz, 1H, aromat. 4-H), 7.64 (t, 1H, aromat. 5-H), 7.77 (s, 1H, 2-H), 7.80 (dd, 1H, 6-H), 11.35 (br. s, 1H, D_2O exchange, NOH); MS (EI, 200°C) *m/z* (%): 198 (8) [$M^{+\bullet}$], 168 (56), 111 (73), 75 (31), 59 (100), 44 (23).

2-Chlorophenylazoamidoxime ***3d***

From 5.1 g 2-chloraniline (40 mmol) and 2.5g (36 mmol) hydroxylamine · HCl. Crystals (chloroform), m.p.: 179°C, yield: 2.1 g (26%). Anal. $C_7H_7ClN_4O \cdot 1/2\ H_2O$ (207.61). IR (KBr) ν [cm^{-1}]: 3491, 3382, 3162, 2853, 1662, 1582, 1537, 1463, 1094, 1059, 1014, 766; ^{1}H-NMR (400 MHz, DMSO-d_6) δ [ppm]: 5.81 (s, 2H, NH_2, D_2O exchange), 7.23 (t, 1H, aromat. 4-H), 7,57 (t, 1H, aromat. 5-H), 7.61 (d, 1H, 3-H), 7.83 (d, *J* = 7.7 Hz, 1H, 6-H), 11.52 (s, 1H, D_2O exchange, NOH); MS (EI, 175°C) *m/z* (%): 198 (8) [$M^{+\bullet}$], 168 (63), 139 (13), 111 (85), 75 (45), 59 (100), 44 (29).

3-Fluorophenylazoamidoxime-semihydrate ***3e***

From 4.44 g 2-chloraniline (40 mmol) and 2.5 g (36 mmol) hydroxylamine · HCl. Crystals (ethylacetate/*n*-hexan), m.p.: 176°C, yield: 1.84 g (24%). Anal. $C_7H_7FN_4O \cdot 1/2\ H_2O$ (197.16). IR (KBr) ν [cm^{-1}]: 3484, 3363, 3075, 2825, 1681, 1598, 1475, 1433, 1239, 1021, 981, 792; ^{1}H-NMR (400 MHz, DMSO-d_6) δ [ppm]: 5.92 (s, 2H, NH_2, D_2O exchange), 7.41 (dt, *J* = 8.4 Hz, *J* = 1.9 Hz, 1H, aromat. 5-H), 7,54 (dd, 1H, aromat. 4-H), 7.64 (dd, *J* = 8.0 Hz, *J* = 5.9 Hz, 1H, 6-H), 7.71 (d, *J* = 8.0 Hz, 1H, 6-H), 11.47 (s, 1H, D_2O exchange, NOH); MS (EI, 200°C) *m/z* (%): 182 (38) [$M^{+\bullet}$], 152 (83), 95 (100), 75 (31), 59 (100), 44 (32).

2-Fluorophenylazoamidoxime ***3f***

From 4.44 g 2-fluoraniline (40 mmol) and 2.5g (36 mmol) hydroxylamine · HCl. Yellow crystals (ethylacetate/*n*-hexane), m.p.: 176°C, yield: 2.20 g (29%). Anal. $C_7H_7FN_4O$ (188). IR (KBr) ν [cm^{-1}]: 3487, 3369, 3090, 2850, 1682, 1553, 1484, 1228, 1106, 1020, 763; ^{1}H-NMR (400 MHz, DMSO-d_6) δ [ppm]: 5.83 (s, 2H, NH_2, D_2O exchange), 7.31 (t, *J* = 7.8 Hz, 1H, aromat. 4-H), 7.48 (t, *J* = 8.5 Hz, 1H, aromat. 5-H), 7.64 (m, 2H, 3-H, 6-H), 11.44 (s, 1H, D_2O exchange, NOH); MS (EI, 35°C) *m/z* (%): 182 (12) [$M^{+\bullet}$], 152 (77), 123 (14), 110 (32), 95 (96), 75 (37), 59 (100), 44 (30).

4-Trifluoromethylphenylazoamidoxime ***3g***

From 6.44 g 4-trifluoraniline (40 mmol) and 2.5 g (36 mmol) hydroxylamine · HCl. Yellow crystals (ethylacetate/*n*-hexane), m.p.: 152°C, yield: 4.15 g (45%). Anal. $C_8H_7F_3N_4O$ (232.2). IR (KBr) ν [cm^{-1}]: 3470, 3385, 3086, 2827, 1668, 1542, 1409, 1323, 1176,

1133, 1066, 1011, 850; ^{1}H-NMR (400 MHz, DMSO-d_6) δ [ppm]: 5.99 (s, 2H, NH_2, D_2O exchange), 7.97 (dd, J = 8.9, J = 4.3 Hz, 4H, aromat. 2-H, 3-H, 5-H, 6-H), 11.61 (s, 1H, D_2O exchange, NOH); MS (EI, 90°C) m/z (%): 232 (7) [$M^{+\bullet}$], 202 (59), 145 (99), 95 (12), 59 (100), 44 (20), 28 (23).

4-Biphenylazoamidoxime ***3h***

From 2.5 g 4-biphenylaniline (14.8 mmol) and 0.97 g (14.0 mmol) hydroxylamine · HCl. Yellow crystals (ethylacetate/*n*-hexane), m.p.: 148°C, yield: 1.2 g (13%). Anal. $C_{13}H_{12}N_4O$ (240.2). IR (KBr) ν [cm^{-1}]: 3465, 3370, 3064, 2830, 1654, 1599, 1551, 1483, 1405, 1006, 849, 767, 695; ^{1}H-NMR (400 MHz, DMSO-d_6) δ [ppm]: 5.89 (s, 2H, NH_2, D_2O exchange), 7.41 (t, J = 7.2 Hz, 1H, aromat. 4′-H), 7.51 (t, J = 7.1 Hz, 2H, aromat. 3′-H, 5′-H), 7.76 (d, 2H, aromat. 2′-H, 6′-H) 7,92 (m, 4H, aromat. 2-H, 3-H, 5-H, 6-H), 11,24 (s, 1H, D_2O exchange, NOH); MS (EI, 90°C) m/z (%): 240 (13) [$M^{+\bullet}$], 210 (51), 168 (55), 153 (100), 59 (100), 43 (15), 28 (10).

4.1.3.7 2,6-Dichlorophenylazoamidoxime ***3i***

From 6.48 g 2,6-dichloraniline (40 mmol) and 2.5 g (36 mmol) hydroxylamine · HCl. Red crystals (ethylacetate/*n*-hexane), m.p.: 180°C, yield 5.0 g (54%). Anal. $C_7H_6Cl_2N_4O$ (232). IR (KBr) ν [cm^{-1}]: 3465, 3365, 3078, 2849, 1668, 1559, 1451, 1432, 1347, 1092, 1027, 792; ^{1}H-NMR (400 MHz, DMSO-d_6) δ [ppm]: 6.01 (s, 2H, NH_2, D_2O exchange), 7.41 (t, J = 8.1 Hz, 1H, aromat. 4-H), 7.62 (d, J = 8.1 Hz, 2H, aromat. 3-H, 5-H), 11.54 (s, 1H, D_2O exchange, NOH; MS (EI, 90°C) m/z (%): 232 (1) [$M^{+\bullet}$], 202 (15), 187 (12), 173 (14), 160 (28), 145 (48), 124 (10), 109 (25), 75 (12), 59 (100), 44 (24).

Biology

Platelet aggregation

The Born test was performed with collagen as inducer as described in the literature [12, 23]. Due to solubility issues, all compounds were assayed with 0.3% DMSO, which did not affect platelet aggregation.

Thrombosis model

The laser thrombosis test was performed as described in detail in the literature [24]. Test compounds were suspended in a solution of arabic gum (1%) and orally administered in a concentration of 60 mg/kg. Control experiments with acetylsalicylic acid (ASS) showed an inhibition of thrombosis in arterioles of 48 ± 10%.

Blood pressure measurements

The blood pressure model has been performed as described in detail in the literature [25].

In-vitro NO-synthase assay

NO formation was measured indirectly by determining nitrite formation via the Griess assay. eNOS and nNOS were obtained from Prof. Dr. B. Mayer (University Graz, Austria), iNOS was purchased from Alexis Biochemicals (Enzo Life Sciences GmbH, Lörrach, Germany). Incubations were performed in assay buffer containing 50 mM triethanolamine pH = 7.5, 0.5 mM NADPH, 5 μM FMN, 5 μM FAD, and 10 μM H_4B. Assay buffer for incubations with eNOS and nNOS additively required 0.5 mM $CaCl_2$ and 10 μg/mL calmoduline. iNOS buffer additionally contained $MgCl_2$ in a final concentration of 1 mM and nNOS buffer additively contained 1 mM CHAPS, 10 mM 2-mercaptoethanol, and 0.5 mM EDTA. Compounds were dissolved in DMSO and diluted to a final concentration of 1 mM in 1.25% DMSO. Some incubation samples additionally contained 0.5 mM L-arginine. Reactions were performed in a final volume of 80 μL and incubated for 30 min at 37°C. Since excessive NADPH can cause low values by reducing the diazonium cation as well as the azo dye, it is necessary to remove NADPH using an additional incubation with 20 μL lactate dehydrogenase (20 U/mL) and 1.6 mM sodium pyruvate [26]. After incubation for 20 min at 37°C, reactions were terminated by adding 50 μL of ice-cold acetonitrile and centrifugation (10 000 × g, 15 min, 4°C). 100 μL of supernatants were mixed with 20 μL of Griess reagent (sulfanilamide 5.4% and *N*-(1-naphthyl)-ethylenediamine 0.9% in 1 N HCl) and the absorption of the formed azo dye was measured at 543 nm against a blank containing all cofactors diluted in assay buffer.

Incubations containing 0.5 mM L-arginine but no test compound were referred to 100%. Specific activities of the applied enzyme sources were 800 nmol min^{-1} mg^{-1} for nNOS, 184 nmol min^{-1} mg^{-1} for eNOS, and >1 nmol min^{-1} mg^{-1} for iNOS.

Detection of HNO and NO

Compounds **1**–**3** were dissolved in phosphate buffer/ethanol (1:1) (3 μM) and argon was flushed through the solution for 20 min. Trifluoroacetoxy-iodosobenzene and Fe^{III}TPPCl were added to obtain a final concentration of 1 mM. The mixtures were incubated for 1 h at 37°C.

The chromatographical determination of N_2O was carried out with a Perkin-Elmer F22 gas-chromatograph, head-space technique, using a Porapak Q-column (Sigma-Aldrich, Germany) and electron capture detector [18]. NO was detected by the chemiluminescence-method as published by Schmidt [19], using an Ansyco NO-analyzer AC 30M (Anysco, Karlsruhe, Germany).

Expression and purification of 6 × His-tagged hDDAH-1

Human DDAH-1 was expressed in *Escherichia Coli* BL21 and purified via Ni-NTA-agarose as described previously [27].

In-vitro hDDAH-1 assay

Inhibition rates of hDDAH-1 were determined as described in detail by Kotthaus *et al*. [28] with the following alterations: Inhibitors were applied in final concentrations of 100 and 500 μM and L-NNMA at a concentration of 200 μM. Due to solubility issues assays contained 5% DMSO.

In-vitro arginase assay

A colorimetric assay was carried out with 0.3 μg of bovine liver arginase in 150 μL of 50 mM Tris buffer pH = 7.4 containing 100 μM $MnCl_2$ and 7 mM L-arginine. Inhibitors were applied at concentrations of 100 and 500 μM. Due to solubility issues, assays contained 5% DMSO. Samples were incubated in a 96-well microplate at 37°C for 30 min, and the reaction was stopped by addition of 200 μL Colder reagent [29]. Microplates were sealed with sealing tape, incubated at 95°C for 20 min and then read at 526 nm.

Reduction of arylazoamidoximes

Compounds were dissolved in DMSO and diluted to a final concentration of 3 mM in 150 μL of 100 mM phosphate buffer pH = 6.3 containing 3% DMSO. Incubations additively contained 1 mM NADH and reactions were started by the addition of 300 μg

enzyme source. Incubations were performed for 30 min at 37°C and reactions terminated by adding 150 µL of ice-cold methanol. Samples were centrifuged (9000 × *g*, 5 min) and analyzed by HPLC-analytics.

Metabolites were separated on a LiChrospher 60 RP-select B (4 × 250 mm; 5 µm; Merck Chemicals, Germany) with a RP-select B 4 × 4.0 mm guard column, autosampler Waters 700 Satellite WISP, Waters 600 Controller, and a Waters 486 absorbance detector set at 320 nm (Waters Corporation, Milford, MA, USA). Eluent: 10 mM octylsulfonate/acetonitrile 73:27, pH = 6.3, and the flow-rate was kept at 1 mL/min. All compounds were separated within 25 min.

The authors have declared no conflict of interest.

References

[1] W. K. Alderton, C. E. Cooper, R. G. Knowles, *Biochem J.* **2001**, *357*, 593–615.

[2] L. Ignarro, F. Murad (Eds.), *Adv. Pharmacol.*, Academic Press, San Diego **1995**.

[3] P. Vallance, J. Leiper, *Nat. Rev. Drug Disc.* **2002**, *1*, 939–950.

[4] P. Ferroni, S. Basili, V. Paoletti, G. Davi, *Nutr. Metab. Cardiovasc. Dis*. **2006**, *16*, 222–233.

[5] L. Raij, *J. Clin. Hypertens. (Greenwich)*, **2006**, *8*, 30–39.

[6] N. Toda, K. Ayajiki, T. Okamura, *Pharmacol. Ther.* **2005**, *106*, 233–266.

[7] T. Ogawa, M. Kimoto, K. Sasaoka, *J. Biol. Chem.* **1989**, *264*, 10205–10209.

[8] J. L. Boucher, C. Moali, J. P. Tenu, *Cell. Mol. Life Sci.* **1999**, *55*, 1015–1028.

[9] S. Ohuchida, *Eur. Pat. Appl. 0325936 A0325932*, **1989**.

[10] J. P. Henrion, M. Phillippe, *Eur. Pat. Appl. US 6179881 B6179881,* **1999**.

[11] G. Longo, *Gazz. Chim. Ital.* **1933**, *63*, 923–926.

[12] G. V. Born, *Nature* **1962**, *194*, 927–929.

[13] B. Clement, *Drug Met. Rev.* **2002**, *34*, 565–579.

[14] B. Clement, T. Kunze, S. Heberling, *Biochem. Biophys. Res. Commun*. **2006**, *349*, 869–873.

[15] J. C. Irvine, R. H. Ritchie, J. L. Favaloro, K. L. Andrews, R. E. Widdop, B. K. Kemp-Harper, *Trends Pharmacol. Sci*. **2008**, *29*, 601–608.

[16] H. Volz, T. Barth, *Justus Liebigs Ann. Chem*. **1989**, 171–175.

[17] H.-J. Duchstein, S. Riederer, *Arch. Pharm.* **1995**, *328*, 317–324.

[18] D. A. Wink, M. Feelisch, in *Methods in Nitric Oxide Research* (Eds.: M. Feelisch, J. S. Stamler), John Wiley & Sons, Chichester, New York, Brisbane, Toronto, Singapur **1996**, pp. 403–412.

[19] H. H. H. W. Schmidt, *Med. Monatsschr. Pharm.* **1994**, *17*, 168–185.

[20] P. A. van Zwieten, *Br. J. Clin. Pharmacol.* **1980**, *10 Suppl 1*, 13S–20S.

[21] J. G. Schantl, W. Türk, *Sci. Pharm.* **1989**, *57*, 375–380.

[22] K. Rehse, S. Bade, A. Harsdorf, B. Clement, *Arch. Pharm.* **1997**, *330*, 392–398.

[23] K. Bethge, H. H. Pertz, K. Rehse, *Arch. Pharm.* **2005**, *338*, 78–86.

[24] K. Rehse, A. Kesselhut, V. Schein, M. Kampfe, B. Rose, E. Unsold, *Arch. Pharm.* **1991**, *324*, 301–305.

[25] K. Rehse, M. Herpel, D. Piechocki, *Arch. Pharm.* **1996**, *329*, 83–86.

[26] J. M. Hevel, M. A. Marletta, *Methods Enzymol.* **1994**, *233*, 250–258.

[27] J. Kotthaus, D. Schade, K. Topker-Lehmann, E. Beitz, B. Clement, *Bioorg. Med. Chem*. **2008**, *16*, 2305–2312.

[28] J. Kotthaus, D. Schade, N. Muschick, E. Beitz, B. Clement, *Bioorg. Med. Chem*. **2008**, *16*, 10205–10209.

[29] M. Knipp, M. Vasak, *Anal. Biochem.* **2000**, *286*, 257–264.

[30] S. Bade, *Ph.D. Thesis*, Freie Universität Berlin, Germany **1995**.

