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Structure–activity relationship of novel and known inhibitors of human dimethylarginine dimethylaminohydrolase-1: Alkenyl-amidines as new leads

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ABSTRACT

Recent studies demonstrated that inhibition of dimethylarginine dimethylaminohydrolase (DDAH) activity could be a new strategy to indirectly affect nitric oxide (NO) formation by elevating N^{ω}-methylated L-arginine (NMMA, ADMA) levels. This approach is an alternate strategy for the treatment of diseases associated with increased NO-concentrations. To date, three classes of potent inhibitors are known: (1) pentafluorophenyl sulfonates (IC_{50} = 16–58 μM, *Pa*DDAH), which are also inhibitors for the arginine deiminase; (2) the most potent inhibitors are based on indolylthiobarbituric acid (IC_{50} = 2–17 μM, *Pa*DDAH), which were identified by virtual modelling; and (3) L-arginine analogs, whose best representative is N^{ω}-(2-methoxyethyl)-L-arginine (IC_{50} = 22 μM, rat DDAH). Based on these known structures, we aimed to develop inhibitors for the human DDAH-1 with improved potency and better relative selectivity for DDAH-1 over NOS. Particularly, the binding pocket of the guanidine-moiety was investigated by screening differently substituted guanidines, amidines and isothioureas in order to collect information on possible binding modes in the active site. All substances were tested in a plate-reader format and HPLC assay and several potent inhibitors were identified with K_i-values varying from 2 to 36 μM, with N^5-(1-iminobut-3-enyl)-L-ornithine (L-VNIO) being the most potent inhibitor of the human DDAH-1 so far described. Besides these potent inhibitors alternate substrates for hDDAH-1 were identified as well.

1. Introduction

As an omnipresent messenger nitric oxide (NO) is involved in various physiological processes, such as regulation of vascular tone, inhibition of platelet aggregation and leukocyte adhesion on the endothelial surface. In addition, it is generated in large quantities by macrophages during host defense and immunological reactions and 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 endogenous NO levels are 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] On the other hand, overproduction of NO can be a serious problem as well, since a number of diseases such as migraine, septic shock or ischemia are associated with elevated NO-concentrations.[7,8] NO is formed in a five-electron oxidation via nitric oxide synthases (NOSs, EC 1.14.13.39) from L-arginine. 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 dimethylarginine dimethylaminohydrolase (DDAH, EC 3.5.3.18) to L-citrulline and either dimethylamine or methylamine (Fig. 1).[9] There are two different isoforms sharing 62% sequence similarity: DDAH-1 is mainly expressed in tissues containing nNOS, whereas DDAH-2 predominantly colocalizes with eNOS, indicating an isoform-specific regulation of NOS activity.[10]

Growing evidence implicates that elevated plasma levels of these physiological inhibitors of NOSs in diseases are associated with impaired NO bioavailability.[15–17] However, reduced ADMA levels were detected in patients with Alzheimer's disease.[18] Thus, inhibition of DDAH activity could be another mechanism to affect NO formation by elevating N^{ω}-methylated L-arginine levels and might present a new promising pharmaceutical strategy to indirectly modulate NO levels.[3] This hypothesis was supported by former studies showing that pharmacological inhibition of DDAH

Abbreviations: *o*-PA, *o*-phthaldialdehyde; L-VNIO, N^5-(1-iminobut-3-enyl)-L-ornithine; *Pa*, *Pseudomonas aeruginosa*; ADMA, N^{ω},N^{ω}-dimethyl-L-arginine; NMMA, N^{ω}-monomethyl-L-arginine; NO, nitric oxide; DDAH, dimethylarginine dimethylaminohydrolase; NOS, nitric oxide synthase; L-NIO, N^5-(1-iminoethyl)-L-ornithine; L-NIL, N^6-(1-iminoethyl)-L-lysine; ENIPO, N^5-[(*E*)-1-iminopent-3-enyl]-L-ornithine; Ethyl-L-NIO, N^5-(1-iminobutyl)-L-ornithine.

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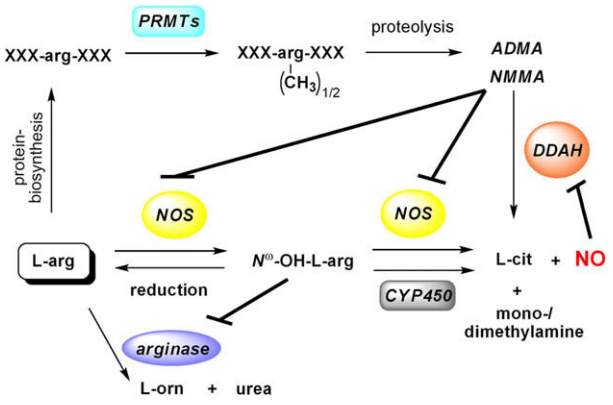


Figure 1. Overview on the NO generating system and its physiological regulation.[11,12] ADMA, N^{ω},N^{ω}-dimethyl-L-arginine; microsomal/mitochondrial reduction, see Clement et al.[13]; CYP450, cytochrome P450, see Boucher et al.[14]; DDAH, dimethylarginine dimethylaminohydrolase; L-arg, L-arginine; L-cit, L-citrulline; L-orn, L-ornithine; NMMA, N^{ω}-monomethyl-L-arginine; NOS, nitric oxide synthase; PRMT, protein-arginine-methyltransferase.

results in an accumulation of ADMA.[19,20] In particular, DDAH-1 inhibition might be a promising option in the treatment of septic shock and certain forms of pain.[21]

Meanwhile, the crystal structures of *Pa*DDAH, bovine DDAH-1 and human DDAH-1 (hDDAH-1) have been published.[21–23] These structures demonstrate the significance of the α-amino acid moiety of L-citrulline and ADMA, respectively, since several H-bonds and ionic interactions are formed within the active site of DDAH. The N^{δ}-atom and the unsubstituted N^{ω}-atom of these amino acids accept one and two H-bonds, respectively.[23] Notably, not much is known about the interaction between the binding cavity and the dimethylated N^{ω}-atom of ADMA. As can be seen in the work of Leiper et al., fitting of N^{ω}-(2-methoxyethyl)-L-arginine (**3a**) in the active site of hDDAH-1 is different from that of L-citrulline, and thus, this different orientation appears to improve affinity to the active site.[21]

Meanwhile, some DDAH inhibitors have been identified: pentafluorophenyl sulfonates, which inhibit the arginine deiminase as well[24] and the mechanism-based inhibitors 2-chloroacetamidin,[25] *S*-nitroso-L-homocysteine[26] and 4-hydroxy-2-nonenal (4-HNE).[27] Furthermore, there are the more potent indolylthiobarbituric acids (**1a**, SR445; IC_{50} = 2 μM, *Pa*DDAH) and L-arginine analogs, with N^{ω}-(2-methoxyethyl)-L-arginine (**3a**, IC_{50} = 22 μM, rat DDAH) inhibiting best.[20,28] The respective methylester exhibits comparative inhibitory potency (IC_{50} = 20 μM, rat DDAH) but is likely to be cleaved by esterases within minutes after oral absorption, yielding **3a** again.

In this work we present our initial attempts to develop more potent inhibitors of the hDDAH-1 and provide new information about structure–activity relationships, particularly with respect to the guanidine binding pocket. To shed light on this binding cavity for the N^{ω}-substituent we investigated several amidines and guanidines and eventually focused on alkenyl-residues. For all compounds a relative selectivity for hDDAH-1 over NOSs was examined.

2. Results and discussion

2.1. Inhibition of recombinant human DDAH-1

All compounds were initially tested in concentrations of 100 μM and 1 mM. K_i-values were determined for those substances that showed inhibition greater than 50% at 1 mM.

In an incipient compound screening we tested various mono-, di- and trisubstituted guanidines. None of these compounds showed an appreciable inhibition of hDDAH-1 (see Supporting information for details). Moreover, in earlier work we found that N^{δ}-methylated L-arginine analogs had no inhibitory potency, underlining the significance of the N^{δ}-atom H-bond.[29] We also screened benzamidines and some known NOS inhibitors like *S*-methylisothiourea[30] and 1400W[31] (for detailed inhibition rates see Supporting information). Besides *S*-methylisothiourea none of these compounds showed potent hDDAH-1 inhibition.

2.1.1. Indolylthiobarbituric acid derivatives

Hartzoulakis et al. identified the indolylthiobarbituric acid SR445 (**1a**) to be the most potent inhibitor of *Pa*DDAH known to date.[28] In that study only IC_{50}-values were given which prompted us to determine K_i-values for the best two inhibitors in this series (**1a**, **1b**) to allow for better comparison between tested compounds (Fig. 2). In conflict to Hartzoulakis et al., these compounds were not soluble in buffer and thus had to be tested in buffer containing 20% DMSO. Despite high DMSO concentration hDDAH-1 activity was not reduced which was confirmed by control incubations. Surprisingly, we could not detect any inhibition of hDDAH-1, although the assay format is similar to that used by Hartzoulakis et al. Although bacterial DDAH is often used as a model for mammalian DDAH there are significant distinctions. A major factor may be that mammalian DDAH is active as a monomer whereas *Pa*DDAH is dimeric. Despite the low sequence similarity between mammalian and *Pa*DDAH (~25%) the active site is highly conserved. Functionally, *Pa*DDAH possesses a broader substrate specificity.[8,23] Interference of the *N*-terminal 6x-His tag of our recombinant hDDAH-1 appears very unlikely. Taken together, **1a** and **1b** appear to be selective towards the bacterial enzyme. Sequence similarity of DDAH-1 is very high (about 92%) within mammalian species, so that we think our results can be transferred to other mammalian species as well.[23] Concluding from these findings, **1a** might be a potent lead for the development of selective bacterial DDAH inhibitors which are of considerable interest, since *Pseudomonas aeruginosa* is a pathogen linked to chronic lung infections and *Pa*DDAH is assumed to play a role in the etiology of lung damage.[28,32]

2.1.2. N^5-(1-iminoalk(en)yl)-L-ornithine derivatives

We assumed that amidines — as bioisosters of guanidines — on the basis of L-ornithine should reveal affinity to DDAH as well. The substance class of such N^5-(1-iminoalk(en)yl)-L-ornithines is well recognized as potent inhibitors of NOSs.[33,34]

Thus, we investigated N^5-(1-iminobut-3-enyl)-L-ornithine (**2a**, L-VNIO), N^5-(1-iminobutyl)-L-ornithine (**2b**, Ethyl-L-NIO), N^5-(1-iminopent-3-enyl)-L-ornithine (**2c**, L-ENIPO), N^5-(1-iminopropyl)-L-ornithine (**2d**, Me-L-NIO) and N^5-(1-iminoethyl)-L-ornithine (**2e**, L-NIO) to explore the influence of chain length and presence of double bonds on hDDAH-1 inhibition. Most of these compounds

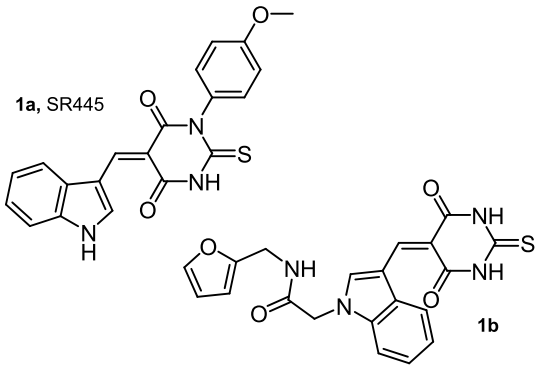


Figure 2. Potent inhibitors of *Pa*DDAH: indolylthiobarbituric acids.

showed potent inhibition of hDDAH-1 in a competitive manner. As can be seen in Table 1, L-VNIO (**2a**) turned out to be the most potent inhibitor of hDDAH-1 identified so far. A C-1 extended derivative (**2c**) of L-VNIO possesses reduced potency. In general, with respect to side-chain length, potency declines in the order Ethyl-L-NIO (**2b**) > Me-L-NIO (**2d**) > L-NIO (**2e**). The relevance of the double bond becomes obvious by comparing **2a** and **2b** because removal of the double bond increases the K_i about 16-fold (Table 1).

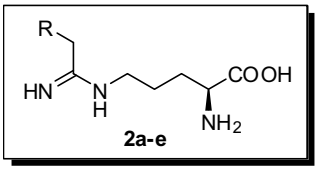
Amino acid main-chain length is essential for the binding of an inhibitor to the active side of DDAH as is reflected by a decrease in potency with C-1 shortened analogs of **3a** and *S*-nitroso-L-cysteine.[20,26] However, increasing main-chain length might be an option since N^{ω},N^{ω}-dimethyl-L-homoarginine is a substrate for DDAH whereas N^{ω},N^{ω}-dimethyl-nor-L-arginine is not.[9,20] Thus, we also tested N^6-(1-iminoethyl)-L-lysine (L-NIL, see Supporting information) which showed reduced activity, ultimately suggesting little margin with respect to alterations of the length of this main-chain.

2.1.3. N^{ω}-substituted L-arginine derivatives

Since **2a** presents the most potent inhibitor of hDDAH-1 identified so far, we attempted to transfer vinyl-like residues to L-arginine to further improve potency. Thus, we examined several novel and known L-arginine derivatives bearing such alkenyl- or alkinyl residues, respectively (Table 2).[35–37] Furthermore, according to the 2-methoxyethyl residue in **3a**, we tested analogs with comparable length anticipating more information about alternate binding options.

From our initial results with N^5-(1-iminoalk(en)yl)-L-ornithine derivatives we conclude that π-electrons improve binding. This assumption is supported by comparing N^{ω}-propyl-L-arginine (**3b**) with N^{ω}-propargyl-L-arginine (**3e**) or N^{ω}-(but-3-enyl)-L-arginine (**3d**) and N^{ω}-allyl-L-arginine (**3c**). It is remarkable that the propyl moiety — with a chain length between that of butenyl and propargyl — shows less inhibitory potency. Interestingly, the propargyl derivative is more potent than the allyl derivative, either demonstrating that affinity is increased by the existence of more π-electrons or pointing to the significance of chain length in N^{ω}-substitution. Derivatives lacking a multiple bond like N^{ω}-(2,2,2-trifluoroethyl)-L-arginine (**3f**) were much less effective, suggesting that hydrophobic interactions are not sufficient. In addition, we examined whether alternate N^{ω}-residues with an appropriate chain length, capable of forming additional H-bonds (acceptor and/or donor), affect affinity to the active site. Both N^{ω}-(2-carbamoylethyl)-L-arginine (**3h**) and N^{ω}-morpholinyl-L-arginine (**3i**), a cyclic analog of **3a**, were clearly less effective than the alkenyl-derivatives.

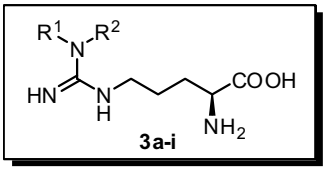
Table 1
Inhibition of hDDAH-1: IC_{50}-values, K_i-values and inhibition rates at 1 mM for N^5(1-iminoalk(en)yl)-L-ornithines (mean values of at least four independent experiments ± SD)



Compound	R	Inhibition at 1 mM[a] (%)	IC_{50}[b] (μM)	K_i[b] (μM)
(**a**)	H_2C	97 ± 3	13 ± 3	2 ± 1
(**b**)	H_3C	78 ± 7	70 ± 21	32 ± 6
(**c**)	H_3C	73 ± 8	79 ± 25	36 ± 3
(**d**)	H_3C	65 ± 8	300 ± 104	145 ± 15
(**e**)	H	24 ± 3		

[a] Determined by hDDAH-1 HPLC assay.
[b] Determined by hDDAH-1 plate-reader assay.

Table 2
Inhibition of hDDAH-1: IC_{50}-values, K_i-values and inhibition rates at 1 mM for N^{ω}-substituted L-arginines (mean values of at least four independent experiments ± SD)



Compound	R^1	R^2	Inhibition at 1 mM[a] (%)	IC_{50}[b] (μM)	K_i[b] (μM)
(**a**)	H_3C–O–	H	89 ± 2	29 ± 7	13 ± 2
(**b**)	H_3C–	H	60 ± 6	283 ± 12	90 ± 5
(**c**)	H_2C=	H	70 ± 3	207 ± 10	58 ± 9
(**d**)	H_2C=	H	71 ± 3	189 ± 10	57 ± 9
(**e**)	HC≡	H	83 ± 7	55 ± 8	17 ± 5
(**f**)	F_3C–	H	41 ± 1		
(**g**)	O_2N–	H	6 ± 4		
(**h**)	H_2N–C(=O)–	H	43 ± 2		
(**i**)			23 ± 3		

[a] Determined by hDDAH-1 HPLC assay.
[b] Determined by hDDAH-1 plate-reader assay.

2.2. Inhibition of nitric oxide synthases

The active sites of NOS isoforms and DDAH are highly similar. Yet, inhibition of NOSs by DDAH inhibitors is undesirable. Hence, we tested the effect of our set of compounds on NOSs activity.

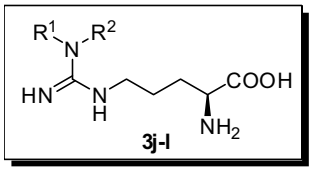
Most of the compounds showed a potent inhibition of all three NOS isozymes (Table 3) which is not surprising since N^5-(1-iminoalk(en)yl)-L-ornithines (**2a–e**) are known as potent inhibitors of NOSs with K_is from 0.1 to 60 μM.[33,34] Moreover, K_is for **3b**, **3c**, **3e** and **3g** were determined to be in the range from 57 nM to 180 μM.[35,38]

Nevertheless, in our assay format N^5-(1-iminopent-3-enyl)-L-ornithine (**2c**, L-ENIPO), N^{ω}-(2-methoxyethyl)-L-arginine (**3a**), N^{ω}-(but-3-enyl)-L-arginine (**3d**), N^{ω}-(2,2,2-trifluoroethyl)-L-arginine (**3f**), N^{ω}-(2-carbamoylethyl)-L-arginine (**3h**) and N^{ω}-morpholinyl-L-arginine (**3i**) were much less effective. Particularly, the low inhibition rates of **2c** and **3d** are remarkable since these compounds showed potent hDDAH-1 inhibition. Concluding from these findings, elongation of this alkenyl residue might be an option to gain selectivity for hDDAH-1 over NOS. This assumption is supported by the work of Rossiter et al., who demonstrated larger residues such as a 2-isopropoxyethyl moiety to be accepted by mammalian DDAH-1.[20] In

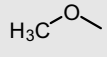
Table 3
Inhibition of NOSs: inhibition rates at 100 μM and 1 mM for N^5-(1-iminoalk(en)yl)-L-ornithines and N^{ω}-substituted L-arginine derivatives (mean values of at least three independent experiments ± SD)

Compound	Inhibition at 100 μM (%)			Inhibition at 1 mM (%)		
	nNOS	eNOS	iNOS	nNOS	eNOS	iNOS
(**2a**)	94 ± 3	45 ± 2	84 ± 1	100 ± 1	88 ± 3	100 ± 1
(**2b**)	34 ± 8	20 ± 6	80 ± 3	100 ± 1	54 ± 3	100 ± 1
(**2c**)	12 ± 11	27 ± 8	6 ± 2	70 ± 8	36 ± 1	21 ± 11
(**2d**)	77 ± 2	20 ± 3	72 ± 2	100 ± 1	85 ± 6	100 ± 1
(**2e**)	75 ± 4	80 ± 5	84 ± 3	100 ± 1	100 ± 1	100 ± 1
(**3a**)	4 ± 12	4 ± 7	0 ± 13	12 ± 8	10 ± 5	0 ± 13
(**3b**)	68 ± 3	15 ± 10	0 ± 3	100 ± 1	55 ± 6	2 ± 15
(**3c**)	68 ± 2	21 ± 5	30 ± 8	100 ± 1	75 ± 5	100 ± 1
(**3d**)	0 ± 11	16 ± 5	0 ± 3	14 ± 4	35 ± 5	40 ± 8
(**3e**)	71 ± 3	20 ± 4	58 ± 4	100 ± 1	87 ± 2	100 ± 1
(**3f**)	17 ± 4	15 ± 1	0 ± 9	34 ± 4	30 ± 6	23 ± 6
(**3g**)	100 ± 1	100 ± 1	91 ± 8	100 ± 1	100 ± 1	100 ± 1
(**3h**)	11 ± 5	15 ± 10	0 ± 7	14 ± 4	37 ± 6	30 ± 6
(**3i**)	0 ± 9	5 ± 7	3 ± 2	17 ± 5	15 ± 11	22 ± 6

Table 4
Substrate studies with hDDAH-1: kinetic properties of L-arginine derivatives



3j-l

Compound	R^1	R^2	K_m[a] (μM)	V_{max}[a] (nmol min^{-1} mg^{-1})
(**j**)		H	130 ± 57	157 ± 7
(**k**)			161 ± 28	60 ± 6
(**l**)		H	106 ± 17	154 ± 24

[a] Determined by hDDAH-1 plate-reader assay.

addition, several investigations revealed that larger residues than the N^{ω}-propyl moiety do not fit into the active site of NOS.[39] This is underlined by the fact that N^5-(1-iminohexyl)-L-ornithine (Butyl-L-NIO) possesses only minor effects on NOS isoforms.[34]

2.3. Substrates for hDDAH-1

Besides inhibitors of hDDAH-1 we also found new substrates for the enzyme. N^{ω}-methoxy-L-arginine (**3j**) and N^{ω}-pyrollinyl-L-arginine (**3k**) turned out to be converted by hDDAH-1 to L-citrulline and presumably the corresponding amines. N^{ω}-methoxy-L-arginine is converted at the same rate as the physiological substrate NMMA (**3l**) whereas N^{ω}-pyrollinyl-L-arginine is a weaker substrate (Table 4). It is to be noted that only few compounds have been identified as substrates for mammalian DDAH, besides the endogenous NMMA and ADMA.[9]

3. Conclusions

So far, inhibition data for different DDAHs was merely described by IC_{50}-values complicating the comparability between separate investigations. In this work, we determined K_i-values for the most potent inhibitors of hDDAH-1. Comparison between these values demonstrates that L-VNIO (**2a**) is the strongest hitherto known inhibitor of hDDAH-1. However, despite the higher affinity of L-VNIO (**2a**) to hDDAH-1, the lack of selectivity over NOSs makes N^{ω}-(2-methoxyethyl)-L-arginine (**3a**) still the best inhibitor so far described.

Interestingly, L-VNIO (**2a**) is the first amidine showing an excellent hDDAH-1 inhibition. Hence, it could be a new promising lead bearing potential for further structural modifications, which will not only be required to increase potency but particularly to gain selectivity over NOS inhibition. In this regard elongation of the alkenyl moiety might be an option. Moreover, as an amidine it also features more drug-like properties than **3a** and provides the opportunity to apply prodrug strategies such as the formation of amidoximes to improve oral bioavailability.[40]

In terms of structure–activity relationship, we have compared different substituents based on N^{ω}-alk(en)yl-L-arginine and N^5-(1-iminoalk(en)yl)-L-ornithine. From these results we conclude that an alken(in)yl-chain of three C-atoms fits best into the binding cavity for the guanidine (amidine) moiety. Further investigations such as cocrystallization of **2a** with hDDAH-1 should be performed to identify the amino acid residues of the enzyme that are responsible for the interaction with the alken(in)yl-chain and to determine the exact orientation of this compound in the active site.

4. Experimental

4.1. General

Commercially available materials were purchased from either Sigma-Aldrich, Fluka, Merck, Alexis Biochemicals or Roth unless otherwise stated. L-NMMA, L-VNIO, N^{ω}-propyl-L-arginine, *S*-ethylisothiourea, L-NIL, 1400W, 2(*S*)-amino-6-boronohexanoic acid were purchased from Alexis Biochemicals. L-NIO and Methyl-L-NIO were purchased from Cayman chemicals. N^{ω}-substituted L-arginine derivatives (**3**) except for N^{ω}-propyl-L-arginine and N^{ω}-nitro-L-arginine were synthesized according to Schade et al.[41] cDNA of hDDAH-1 was obtained from RZPD (Berlin, Germany), Ethyl-L-NIO (**2b**) and L-ENIPO (**2c**) were obtained from BogaR Laboratories LLC (Alpharetta, USA). *N*-(2-furanylmethyl)-3-[(tetrahydro-4,6-dioxo-2-thioxo-5(2H)-pyrimidinylidene)methyl]-1H-indole-1-acetamide (**1b**) was purchased from Asinex and SR445 was purchased from Vitas-M Laboratory Ltd.

Neuronal NOS (rat, recombinant), inducible NOS (mouse, recombinant) and endothelial NOS (bovine, recombinant) were purchased from Alexis Biochemicals.

4.2. Analytical reversed phase HPLC

Formed L-citrulline was detected by RP-HPLC using *o*-PA precolumn derivatization. Metabolites were separated at 30 °C on a Nova-Pak RP_{18} (4 × 150 mm, VDS Optilab) 4 μm with a Phenomenex C18, 4 × 3.0 mm guard column, autosampler Waters 717plus, Waters 600 Controller and a Waters 470 scanning fluorescence detector, set at λ_{ex}: 338 nm, λ_{em}: 425 nm. Gradient system: eluent A consisted of 86% 10 mM potassium phosphate buffer (pH 4.65), 8% acetonitrile, 6% methanol; eluent B consisted of 40% acetonitrile, 30% methanol, 30% *aqua bidest*.

For derivatization the autosampler was set to mix 14 μL of *o*-PA reagent with 10 μL of sample with a reaction time of three minutes at room temperature before injection. *o*-PA reagent was prepared by dissolving 50 mg *o*-PA in 1 mL methanol, followed by adding 9 mL of 0.2 M potassium borate buffer pH 9.4 and 53 μL of 2-mercaptoethanol.

The following elution conditions were used: flow-rate was kept at 1 mL/min; 0–2 min, isocratic with 100% eluent A; 2–3.5 min, linear gradient to 10% eluent B; 3.5–12 min, isocratic with 90% eluent A and 10% eluent B; 12–16 min, linear gradient to 75% eluent B; 16–25 min, isocratic with 25% eluent A and 75% eluent B; 25–

28 min, linear gradient to 100% eluent A; 28–35 min, reequilibration with 100% eluent A.

4.3. Expression and purification of 6x-His-tagged hDDAH-1

Human DDAH-1 was expressed in *Escherichia coli* BL21 and purified via Ni–NTA-agarose (Qiagen) as described previously.[29]

4.4. In vitro hDDAH-1 HPLC assay

A standard HPLC assay consisted of 2 μg recombinant hDDAH-1 in 75 μL 50 mM potassium phosphate buffer pH 7.4 and L-NMMA in a final concentration of 200 μM. For substance screening the inhibitors were applied at concentrations of 100 μM and 1 mM. Due to solubility problems the indolthiobarbituric acid derivatives were tested in 50 mM potassium phosphate buffer pH 7.4 containing 20% DMSO.

Incubations were performed at 37 °C for 30 min, reactions were stopped by adding 75 μL of ice-cold acetonitrile. Samples were centrifuged at 12,000g for 10 min and analyzed for L-citrulline by RP-HPLC using *o*-PA precolumn derivatization.

4.5. In vitro hDDAH-1 plate-reader assay

A colorimetric assay was carried out in 150 μl 50 mM potassium phosphate buffer pH 7.4 with 4 μg of recombinant hDDAH-1 and varying concentrations of substrate and inhibitor (see Sections 4.5.1–4.5.3). 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.[42] Microplates were sealed with sealing tape, incubated at 95 °C for 20 min and then read at 450.5 nm.

4.5.1. K_i-determination

For K_i-determination hDDAH-1 was incubated with five different concentrations (50, 100, 300, 700 and 1250 μM). Each dilution was incubated each inhibitor in five different concentrations and one without inhibitor as a control. K_i-values were calculated using SigmaPlot 8.0 (SPSS Inc.) and Microsoft Excel.

4.5.2. IC_{50}-determination

For IC_{50}-determination hDDAH-1 was incubated with 300 μM NMMA and five different concentrations of inhibitor. IC_{50}-values were calculated using SigmaPlot 8.0 (SPSS Inc.).

4.5.3. K_m-determination for alternate substrates

K_m-values for alternate substrates were determined by incubating hDDAH-1 with N^{ω}-methoxy-L-arginine (**3j**) and N^{ω}-pyrollinyl-L-arginine (**3k**) in concentrations of 50, 75, 100, 200, 500, 750, 1000, 2500 and 5000 μM. K_m-values were determined using SigmaPlot 8.0 (SPSS Inc.).

4.6. In vitro NOS assay (oxyhemoglobin assay)

The activity of the NOS isoforms was determined by the oxyhemoglobin assay as described previously.[43] Oxyhemoglobin was prepared as described in detail elsewhere.[44] The standard assay for nNOS and eNOS was carried out at 37 °C in a total volume of 125 μl containing 1 mM L-arginine, 100 μM NADPH, 10 μM BH_4, 10 μg/ml calmodulin, 1 mM $CaCl_2$ and 170 μM DTT in 50 mM HEPES, pH 7.4. In case of the iNOS, calmodulin was not added and $CaCl_2$ was replaced by 1 mM $MgCl_2$.

The inhibitors were added in concentrations of 100 μM and 1 mM. After addition of 5 μM oxyhemoglobin the reaction was initiated with NOS and the formation of methemoglobin measured at 401 nm by using a Varian cary UV–vis spectrophotometer. As an internal reference the extinction at the isosbestic point (411 nm) was monitored.

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Supplementary data

Details of screened compounds and hDDAH-1 inhibition data. Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmc.2008.10.058.

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