Journal of
Medicinal Chemistry

Brief Article

Discovery of *N*-(4-Aminobutyl)-*N*'-(2-Methoxyethyl)guanidine as the First Selective, Non-Amino Acid, Catalytic Site Inhibitor of human dimethylarginine dimethylaminohydrolase-1 (*h*DDAH-1)

Ina Lunk, Felix-A. Litty, Sven Hennig, Ingrid R Vetter, Jürke Kotthaus, Karin S. Altmann, Gudrun Ott, Antje Havemeyer, Carmen Carrillo García, Bernd Clement, and Dennis Schade

J. Med. Chem., **Just Accepted Manuscript** • DOI: 10.1021/acs.jmedchem.9b01230 • Publication Date (Web): 16 Dec 2019

Discovery of *N*-(4-Aminobutyl)-*N*′-(2-Methoxyethyl)guanidine as the First Selective, Non-Amino Acid, Catalytic Site Inhibitor of human dimethylarginine dimethylaminohydrolase-1 (*h*DDAH-1)

Ina Lunk,[1‡] Felix-Alexander Litty,[1‡] Sven Hennig,[3] Ingrid R. Vetter,[2] Jürke Kotthaus,[1] Karin S. Altmann,[1] Gudrun Ott,[1] Antje Havemeyer,[1] Carmen Carrillo García,[1] Bernd Clement,[1] Dennis Schade[1,2,4]*

[1] Department of Pharmaceutical and Medicinal Chemistry, Christian-Albrechts-University Kiel, Gutenbergstr. 76, D-24118 Kiel (Germany)

[2] Max-Planck-Institute of Molecular Physiology, Otto-Hahn-Str. 11, D-44227 Dortmund (Germany)

[3] Department of Chemistry & Pharmaceutical Sciences, VU University Amsterdam, De Boelelaan 1108 (The Netherlands)

[4] Partner site Kiel, DZHK, German Center for Cardiovascular Research, D-24105 Kiel (Germany)

DDAH, nitric oxide, guanidine, amidine, prodrug

ABSTRACT: *N*-(4-aminobutyl)-*N*'-(2-methoxyethyl)guanidine (**8a**) is as a potent inhibitor targeting the *h*DDAH-1 active site (K_i = 18 µM) and derived from a series of guanidine- and amidine-based inhibitors. Its non-amino acid nature leads to high selectivities towards other enzymes of the nitric oxide-modulating system. Crystallographic data of **8a**-bound *h*DDAH-1 illuminated a unique binding mode. Together with its developed *N*-hydroxyguanidine prodrug **11**, **8a** will serve as a most widely applicable, currently available pharmacological tool to target DDAH-1-associated diseases.

INTRODUCTION

Human dimethylarginine dimethylaminohydrolase-1 (*h*DDAH-1) has gained considerable attention as a drug target for pharmacological intervention of several diseases in recent years. The enzyme catalyzes the hydrolytic degradation of N^{ω}-methylated L-arginines such as monomethyl-L-arginine (NMMA) and N^{ω},N^{ω}-dimethyl-L-arginine (ADMA).[1, 2] These methylarginines can act as endogenous inhibitors of all three isoforms of nitric oxide synthase (NOS) thereby reducing the formation of nitric oxide (NO).

Hence, targeting DDAH is an alternative strategy to indirectly affect NO formation in situations where elevated NO levels contribute to pathophysiology, e.g. septic shock, certain forms of pain (e.g., migraine), pulmonary fibrosis, inflammatory and neurodegenerative disorders or tumor angiogenesis.[2-5] In the latter context, increased expression and/or activity of DDAH-1 has been found in different cancer types and linked to tumor aggressiveness, for example in prostate cancer.[6, 7] Specific targeting of the DDAH-1 is attractive as this isoform is not much expressed in immune cells (e.g., macrophages) and endothelial cells, and thus, does not significantly contribute to vascular NO production which has been largely attributed to DDAH-2.[2]

Given its clinical relevance, it comes as a surprise that rather few DDAH inhibitor chemotypes have been reported to date, with the majority representing substrate analogs.[8] A relatively small-sized substrate binding pocket with restricted ligand access could be possible reasons. The very first described candidates (2005) were the promiscuous, covalently reactive chloroacetamidine as well as *S*-nitroso-L-*homo*-citrulline.[9, 10] In the same year, the Leiper group disclosed the currently most widely used, potent, reversible and selective *h*DDAH-1 inhibitor L-257 (**1**).[11] This compound served as a great starting point for further development and subsequently advanced to the recently reported ZST-316 (**2**).[12] Here, bioisosteric replacement of the α-carboxy group by an acylsulfonamide improved potency by 13-fold (K_i = 1 µM) and showed efficacy in breast cancer models *in vitro*.[13]

Within our own long-standing efforts in designing small molecule inhibitors of *h*DDAH-1, we synthesized and tested several amidine- and guanidine-based inhibitors and identified N^5-(1-iminobut-3-enyl)-L-ornithine (V-NIO, **3**, K_i = 2 µM) as a highly potent candidate.[14] Later, the Fast group explored the amidines toward NOS/DDAH dual inhibitory profiles and characterized their (reversible) binding modes by X-ray crystallography.[15] Building on these structure-activity relationships (SARs), irreversible inhibitors were designed with a chloroacetamidine as the covalent warhead (e.g., Cl-NIO, **4**).[16, 17] Only few more chemotypes have been reported for *h*DDAH inhibition such as 4-chloropyridine, ebselen, 4-hydroxy-2-nonenal or PD-404182, all with questionable selectivities.[18-20]

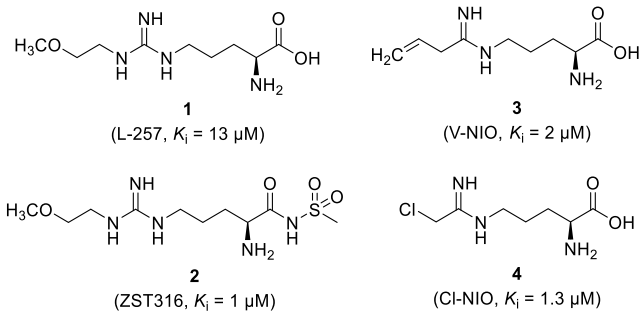


Figure 1. Chemical structures and K_i values of known substrate-based *h*DDAH-1 inhibitors harboring either guanidine (**1**, **2**) or amidine functionalities (**3**, **4**).

Thus, there is a great need for novel modalities with lead-like qualities which we addressed in the present study. Since amidine **3** did not exhibit a desirable selectivity over other key enzymes of the NO regulating system, i.e. NOSs and arginase,[21] but guanidine **1** did, we revisited the structural basis for this phenomenon. Here, for the first time, we could show that the α-carboxy group is dispensable for potent *h*DDAH-1 inhibition. This finding potentially opens avenues to the design of lead-like candidates which we exemplified with a viable prodrug approach.

RESULTS AND DISCUSSION

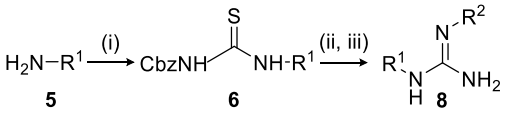
Chemistry. Diversely substituted guanidine-based inhibitor candidates were accessible *via* previously reported methods (Scheme 1A).[22, 23] Briefly, preparation of Cbz-protected thioureas (**6**) followed by EDCI-mediated desulfurization and reaction with desired amines furnished guanidines (**8**) after final deprotection with TFA/thioanisol in moderate to good overall yields (30-88%). The *N*-hydroxylated prodrug candidate of guanidine **8a** was prepared in a similar fashion (Scheme 1B). From *O*-THP-protected 2-methoxyethylthiourea **9**, the *N*-hydroxyguanidine scaffold was built up with *tert*-butyl(4-aminobutyl)carbamate (**10**) in the presence of EDCI, followed by simultaneous deprotection of both THP- and Boc-protecting groups with HCl/dioxane.

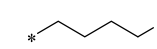
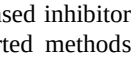
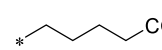
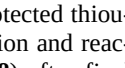
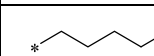
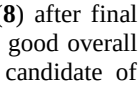
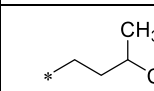
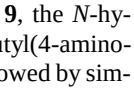
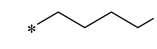
The synthesis of novel amidine-based DDAH inhibitors largely built on strategies described for the preparation of **3** and similar NOS inhibitor candidates.[24, 25] As depicted in Scheme 2, this chemistry employed various imidates (**14**) that were reacted with the desired amines (**15**). A final deprotection step of either/or Boc- and *t*Bu-ester groups with TFA in dichloromethane furnished amidines **16** in moderate to good overall yields (30-76%).

Inhibition profiles: DDAH-1, NOSs, arginase. The most potent *h*DDAH-1 inhibitors belong to the large group of substrate mimetics, especially N^{ω}-substituted L-arginine analogues and N^{5}-(1-iminoalk(en)yl)-L-ornithine derivatives.[8] As outlined in the introduction, specific targeting of *h*DDAH-1 represents an attractive option to perturb NO production in a tissue-selective fashion while not interfering with physiological NO functions. To keep this potential pharmacological asset, activities on revelant inhibitor off-targets need to be minimized. This mainly includes selectivities over NOSs and arginases, both of which share very similar substrates, inhibitors and active site topologies.[1, 15] The distinct selectivity profiles of **1** and **3** on theses enzymes were deemed a promising starting point. Interestingly, L-arginine analogue **1** ($K_{i\,(hDDAH\text{-}1)}$ = 13 µM) already exhibits an excellent selectivity profile over the other arginine converting enzymes. A key responsible feature seems to be the N^{ω}-(2-methoxyethyl)-substituent that is not well-tolerated by NOSs due to size-restrictions in the guanidinium-binding pocket.[15] In sharp contrast, amidine analogue **3** is even more potent against *h*DDAH-1 (K_i = 2 µM) but completely lacks selectivity (Table 1).[21] The question was which pharmacophoric elements contribute most significantly to potency and selectivity? The 2-methoxyalkyl-substituent, a guanidine- or an amidine-scaffold? Therefore, systematic combinations of *N*-(2-methoxyalkyl)- and alkenyl-groups with either guanidine- (Table 1) or amidine-based (Table 2) compounds were tested. At the same time, modifications of the amino acid side chain were realized to revisit the influence of the α-amino acid moiety.

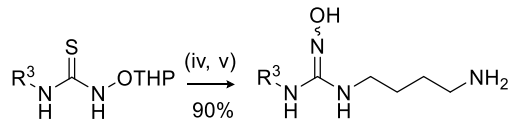
Scheme 1. Synthesis of guanidine-based inhibitors (8) (A), including the *N*-hydroxylated prodrug candidate 11 (B).[a]

A



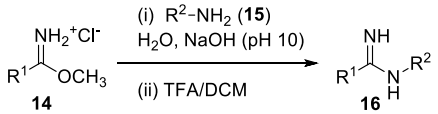
entry	R^1	R^2	overall yield (%)
8a			87
8b			80
8c			88
8d			88
8e			30

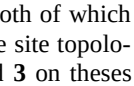
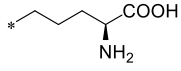
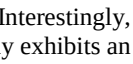
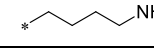
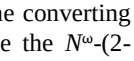
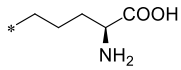
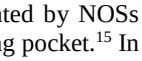
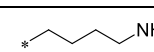
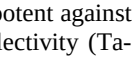
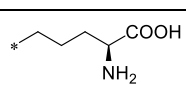
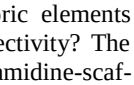
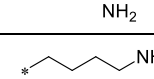
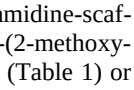
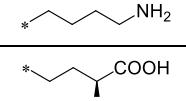
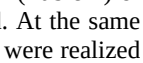
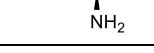
B



[a] Reagents: **A)** (i) *N*-benzyloxycarbonyl isothiocyanate, (ii) R^2-NH_2 **7**, (iii) TFA/thioanisole; **B)** (iv) *tert*-butyl(4-aminobutyl)carbamate **10**, (v) HCl, dioxane, R^3 = 2-methoxyethyl.

Scheme 2. Synthesis of amidine-based inhibitors (16).[a]



entry	R^1	R^2	overall yield (%)
16a			65
16b			67
16c			67
16d			63
16e			75
16f			52
16g			30
16h			76

[a] Detailed information on the synthesis of novel imidates **14** and amidines **16** can be found in Supporting Information.

Data shown in Table 1 revealed for *N*-(2-methoxyethyl)-guanidine derivatives **8a-8d** that the amino acid group cannot be replaced by methyl- (**8c**) or isopropyl- (**8d**) as such modifications led to massively decreased potency or lack of activity, respectively. However, removal of the carboxylic acid moiety (**8a**, K_i = 18 ± 6 µM) was tolerated and retained potent DDAH inhibition in about the same range as the original amino acid **1** (K_i = 13 ± 2 µM). Most importantly, both compounds showed comparable characteristics regarding selectivity over NOS isoenzymes and arginase. This finding was surprising and indicated that the *N*-(2-methoxyethyl)-guanidine group contributes significantly to DDAH binding affinity. To underline the importance of the 2-methoxyethyl-substituent, we replaced it with a similarly sized butenyl-residue (**8e,f**), mimicking the potent amidine N^5-(1-iminobutenyl)-ornithine (**3**). Both the amino acid (**8f**, K_i = 57 µM)[21] and decarboxylated (**8e**, K_i = 59 µM) compounds showed decent and comparable inhibitory activities on *h*DDAH-1. These results confirmed that the α-carboxylic acid is indeed dispensable for DDAH-1 inhibition. However, the overall selectivity profiles of **8e,f** were inferior to **1** and **8a**, highlighting the value of the 2-methoxyalkyl group. Still, it should be noted that both decarboxylated **8a** and **8e** tended to be more selective than their amino acid congeners.

Next, we attempted transferring these findings to an amidine-based scaffold (Table 2). Since the 2-methoxyalkyl-group turned out essential, we systematically searched for a 'sweet spot' in length of this substituent (see **16a-f**). Both the amino acid as well as decarboxylated analogues were synthesized and tested to recapitulate results with the guanidino compound series **8**. As shown in Table 2, all α-amino acids derivatives were moderate to highly potent inhibitors of DDAH-1, with **16c** (K_i = 9 µM) representing the best candidate. In contrast to guanidines **1** and **8**, omitting the α-carboxy group in amidines **16** corrupted DDAH inhibition by 1-2 orders of magnitude in all cases. Since activity appeared to drop with longer methoxyalkyl-residues, compound **16g** was designed and tested. Here, we tested whether methylene-extension in the methoxyalkyl-residue can be compensated by shortening the amino acid side chain (i.e., *nor*-L-ornithine). No DDAH inhibition was detected for **16g** which is in general agreement with previous work on *nor*-arginine or *nor*-ornithine analogues.[11] This data further emphasized the particular importance of an intact α-amino acid group-centred binding mode of amidine-based compounds. To complete this SAR aspect, we also tested a decarboxylated version of **3** (i.e., **16h**) which was almost 400-fold less potent in direct comparison ($K_{i\,(hDDAH\text{-}1)}$ = 768 µM).

Together, our DDAH inhibition data suggested that omitting the α-carboxy group is tolerated only by guanidines but not by amidines. It also implied a predominant role for a 2-methoxyethyl-substituent. In this regard, 2-methoxyethyl-amidine **16c** performed similar to **1** as a *h*DDAH-1 inhibitor, but turned out much less selective towards NOSs and arginase (see Table 2). Promiscuous inhibition of these enzymes was a general observation for all amidines presented herein.

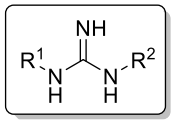
***h*DDAH-1 crystal structure and binding mode.** Systematic exploration of distinct substitution patterns of guanidines **8** and amidines **16** revealed important structural elements that contributed to high affinity for DDAH-1. A surprising finding was that the α-COOH appeared only dispensable for the guanidine-type of inhibitors as exemplified with pairs **1/8a** or **8f/e** compared to amidines **16c/d**. Thus, we determined the crystal structures for ligand-free apo-*h*DDAH-1 (solved at 2.41 Å, pdb 6szq) as well as *h*DDAH-1 in complex with the best inhibitor candidate **8a** (solved at 1.76 Å, pdb 6szp). Overlay of both structures showed a conserved overall structure of *h*DDAH-1, that is comparable to all known structures (Fig. 2A, Fig. S4). Superposition of **8a** (this work, pdb 6szp)- and **1**-bound *h*DDAH-1 (pdb 2jaj) shows almost identical binding modes of both inhibitors (Fig. 2B).

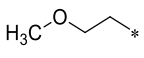
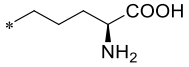
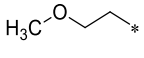
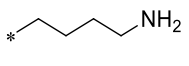
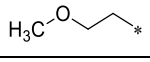
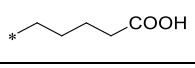
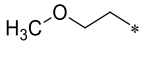
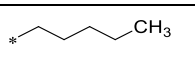
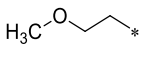
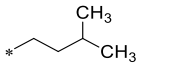
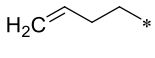
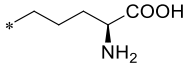
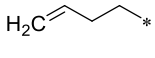
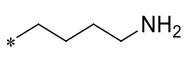
A detailed comparison of guanidine- (**1** and **8a**), amidine- (e.g., **L-IPeO**, pdb 3p8p)[26] and urea (i.e., natural catalytic DDAH product L-citrulline, pdb 2jai)[3]-based DDAH-ligands provided interesting insights into distinct binding modes (Fig. 2C): All ligands have the primary amine of the original α-amino acid moiety locked into place by tight interactions with the side chain of Asp73 and backbone interactions via carbonyl oxygens of Leu30 and Val268. This underlines the particular relevance of the α-NH_3^+ group for potent binding and is likely due to optimal geometric orientation of all participating H-bond partners. However, the α-carboxy groups in both **L-IPeO** and L-citrulline point towards Arg145, whereas α-COOH of **1** is twisted away from Arg145 which in turn is rotated outwards not undergoing one interaction anymore. The exact same twisting of Arg145 is observed for bound **8a**. Given the fact that the butyl chains in **1** and **8a** align very well within the *h*DDAH-1 binding pocket, it appears that a quite specific and significant orientation of these two inhibitors originates from their guanidine moieties. Even the energetically less favoured gauche conformation of the butyl chain is tolerated due to the strong binding via the guanidino and α-amino groups. In contrast, butyl chains in amidine-based inhibitors adopt anti conformations suggesting weaker binding contributions. This putative guanidine-triggered butyl chain orientation seemed to make an interaction with Arg145 dispensable, and thus, the necessity of an α-COOH group for potent binding.

Following this hypothesis, we questioned which contributions of the guanidine are responsible for this phenomenon and how does this binding pattern differ from other ligands? The crystal structure of **1**- and **8a**-bound *h*DDAH-1 showed that the outwards pointing *NH* in **1**/**8a** is interacting with the side chain-carboxylate of Asp79 (Fig. 2C). The aminobutyl-substituted guanidino-*NH* additionally contacts Asp79. The second set of interactions holds the methoxyethyl-substituted (distal) guanidino-*NH* on the opposite side in place via side chain- and backbone carbonyl-oxygens of Asp269 (Fig. 2C, Fig. S6/7). Thus, Asp79 and Asp269 together form two clamps causing a tight fixation of the guanidino group (Fig. 2D). As a result, the relative orientations of both substituents (i.e., 2-methoxyethyl and 4-aminobutyl) are well-predestined. This is in sharp contrast to the **L-IPeO** and L-citrulline binding modes, where the respective amidino or urea group cannot be fixated by Asp79/269 in the same fashion (Fig. 2C). The consequence is greater flexibility of both the pentyl chain (**L-IPeO**) as well as the amino acid side chain (**L-IPeO,** L-citrulline). We believe that the high basicity of a guanidine, compared to an amidine or urea, further contributes to tight binding due to stronger electrostatic interaction within this acidic site (see Fig. S8). This strong positioning of the guanidino group within the two Asp clamps induces a rotation of His173 out of its apo-position, eventually causing an outward twist of Arg145. This effect can neither be observed for **L-IPeO** nor L-citrulline (Fig. 2C).

Lacking the guanidine group-mediated pre-orientation of the two alkyl-substituents in an amidine-based inhibitor seems to create a higher degree of conformational freedom enabling interaction of the α-carboxy group with Arg145. Here, this opportunity becomes vital to ensure potent binding and – once lost due to a missing α–COOH group – results in loss of activity against *h*DDAH-1 (see Table 2, all amidine-based inhibitors).

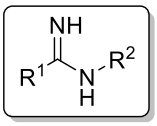
Table 1. Selectivity profiles of guanidine-based DDAH inhibitors.

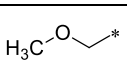
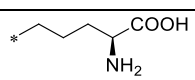
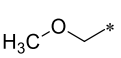
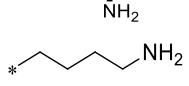
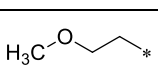
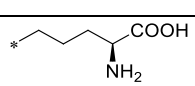
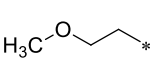
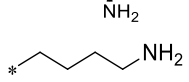
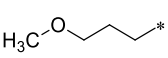
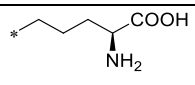
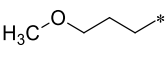
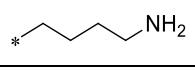
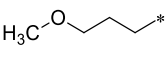
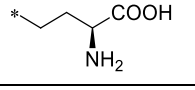
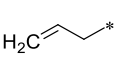
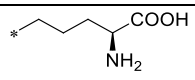
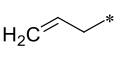
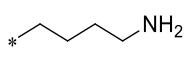


			*h*DDAH-1	Arginase	nNOS	eNOS	iNOS
entry	R^1	R^2	K_i (μM)	% inhibition at 1 mM			
1[a]			13 ± 2	29 ± 6	12 ± 8	10 ± 5	0 ± 13
8a			18 ± 6	11 ± 6	0 ± 8	13 ± 5	0 ± 13
8b			489 ± 16	35 ± 4	5 ± 4	13 ± 4	2 ± 6
8c			2815 ± 946	15 ± 4	0 ± 8	0 ± 4	21 ± 4
8d			2763 ± 714	20 ± 2	0 ± 1	19 ± 10	4 ± 12
8f[a]			57 ± 9	70 ± 21	14 ± 4	35 ± 5	40 ± 8
8e			59 ± 3	0 ± 12	5 ± 5	35 ± 17	23 ± 6

K_i -values are mean values of at least two independent experiments ± SD**.** Inhibition data at 1 mM derived from at least three independent experiments (mean ± SD). [a] Values taken from Kotthaus *et al.* (2012)[21]

Table 2. Selectivity profiles of amidine-based inhibitors



			*h*DDAH-1	Arginase	nNOS	eNOS	iNOS
entry	R^1	R^2	K_i (μM)	% inhibition at 1 mM			
16a			73 ± 6	22 ± 2	93 ± 2	89 ± 4	75 ± 5
16b			983 ± 19	21 ± 2	78 ± 1	44 ± 19	42 ± 8
16c			9 ± 1	43 ± 1	65 ± 1	54 ± 18	50 ± 7
16d			1446 ± 91	22 ± 2	19 ± 23	28 ± 6	16 ± 5
16e			156± 9	20 ± 1	35 ± 13	29 ± 19	26 ± 13
16f			n.d.[b]	14 ± 2	7 ± 13	36 ± 6	15 ± 4
16g			n.d.[b]	n.d.	n.d.	n.d.	n.d.
3[a]			2 ± 1	76± 10	100 ± 1	88 ± 3	100 ± 1
16h			768 ± 4	6 ± 5	99 ± 1	56 ± 8	100 ± 1

K_i -values are mean values of at least two independent experiments ± SD**.** Inhibition data at 1 mM derived from at least three independent experiments (mean ± SD).[a] Values taken from Kotthaus *et al.* (2012)[21]; [b] n.d. = not determined, when inhibition values were below 20% at 1 mM.

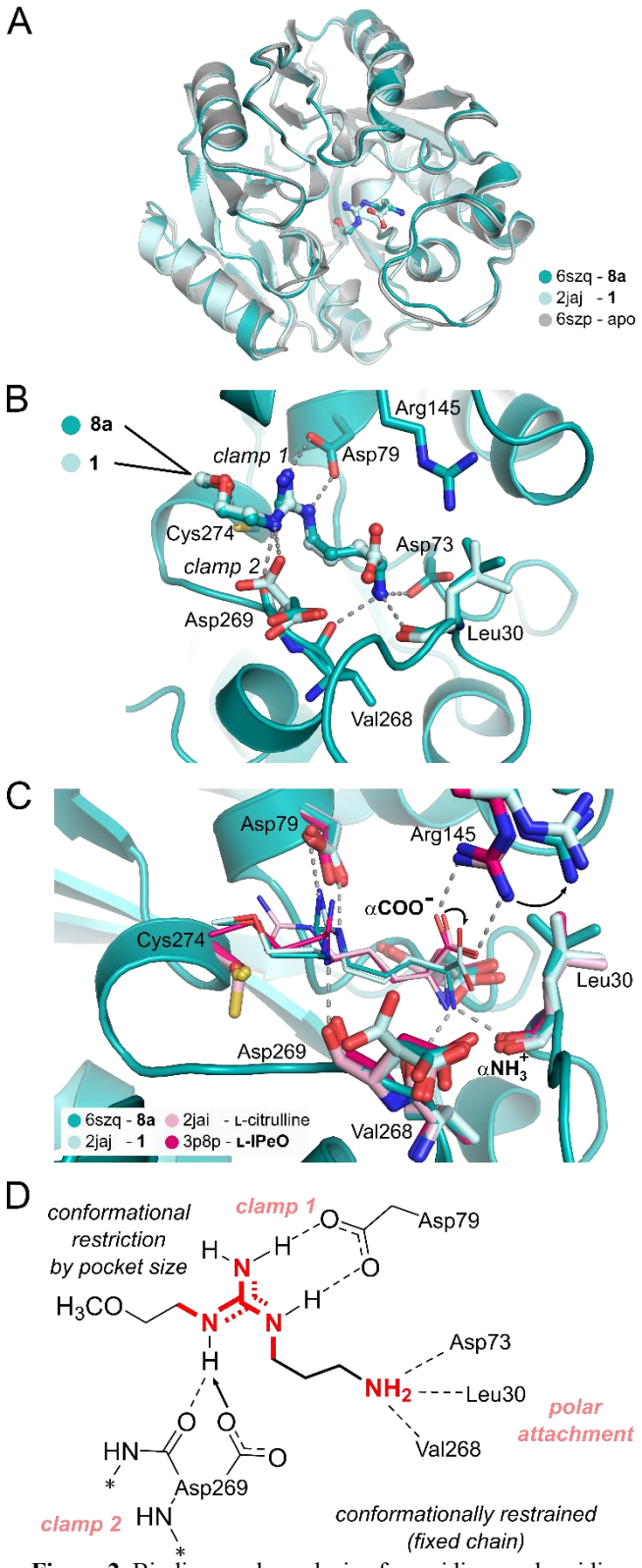
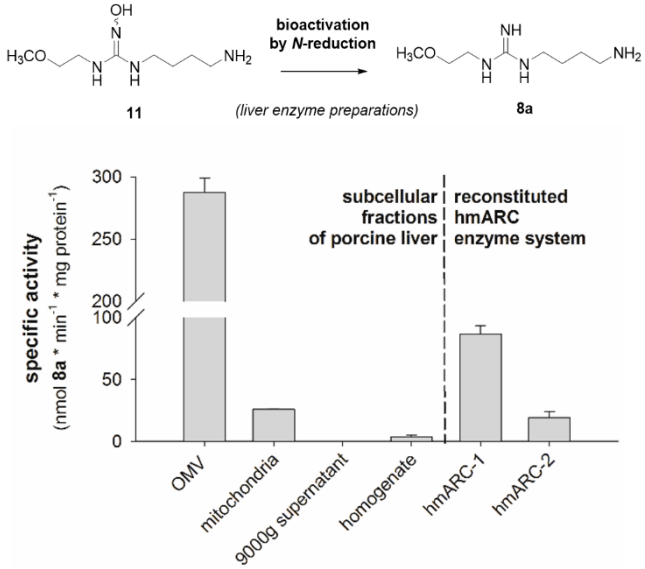


Figure 2. Binding mode analysis of guanidine- and amidine-types of *h*DDAH-1 inhibitors. **A)** Superposition of crystal structures of ligand-free (apo) or **1**- or **8a**-bound *h*DDAH-1; **B)** Binding modes of superimposed **8a** and **1**, dashed lines indicate interacting residues; **C) 8a**-bound *h*DDAH-1 with superimposed **1** (= guanidines), L-citrulline (= urea) and **L-IPeO** (= amidine). **D)** Schematic view on **8a** binding mode, illustrating contributions by active site amino acids.

Future studies will have to address the particular importance of the 2-methoxyethyl-substituent as there did not seem to be direct contacts in close proximity or water-mediated interactions. The closest possible contact would be Cys274 in ca. 3.6 Å distance (Fig 2D). Moreover, it would also be interesting to see whether conformational restrictions within the butyl chain can rescue potency and selectivity of amidine analogs.

Bioactivation of guanidine-prodrug candidate 11. Compound **8a** exemplified that the α-carboxy group is dispensable for potent DDAH-1 inhibition while conveying the molecule with high selectivity over other enzymes of the NO modulating system. These features highlight prime qualities for lead optimization. In this regard, an opportunity for oral administration is generally desirable. Due to the zwitterionic structure of **1,** absorption would only be mediated by active uptake via amino acid transporters. The preferred route of absorption after oral intake would be by passive diffusion. Although this is feasible for the primary amine of **8a**, its guanidine functionality would be permanently charged under physiological conditions, preventing significant membrane transport by passive diffusion. Therefore, we applied a well-established prodrug approach and prepared *N*-hydroxyguanidine **11**. We and others have demonstrated that such *N*-hydroxylated guanidines, similar to amidoximes as prodrugs of amidines, are effectively bioactivated by the mARC-containing *N*-reductive enzyme system *in vitro* and *in vivo*.[27]

Figure 3. *In vitro* bioactivation of *N*-hydroxyguanidine **11** to **8a**.[a]



[a] Data represent means of biological triplicates ± SD. OMV, outer mitochondrial membrane vesicles.

Here, we could show that *N*-hydroxyguanidine **11** is very efficiently converted to the active principle **8a** (Fig. 3). Systematic exploration of distinct subcellular liver fractions revealed highest reduction rates of **11** in outer mitochondrial membrane vesicles (OMV) followed by mitochondria and liver homogenate. Furthermore, incubations with reconstituted, heterologously expressed human mARC-1 and -2 isoenzymes confirmed that **11** is a very good substrate of this *N*-reductive enzyme system. The determined specific activities are in a similar range as other reported substrates, such as benzamidoxime (served as positive control),[28] sulfamethoxazole hydroxylamine [29] or the orphan drug upamostat.[30] Based on this data, there is a

high probability for decent peroral bioavailability of **11**. A general concern is chemical stability of *N*-hydroxyguanidines.[1] However, we found that **11** was very stable at different pHs over 24 hours at 37°C (see Fig. S3). Finally, both the guanidine **8a** and its prodrug **11** revealed an excellent profile regarding cell toxicity/viability (see Supp Info 2.4), further underlying their utility as a pharmacological toolset.

CONCLUSION

The human DDAH-1 has emerged as an attractive drug target in the past two decades, and recently gained particular attention in the context of tumor angiogenesis and progression. It has been discussed that indirect regulation of NO production via DDAH-1 inhibition would represent a safer option compared to the use of NOS inhibitors. However, only few agents with lead-like qualities have been described to date. Through rational design, synthesis, biochemical profiling and crystallization, we dissected key structural requirements for high affinity-binding to *h*DDAH-1 while ensuring a desired selectivity towards other key enzymes of the NO-modulating system.

These efforts culminated in the discovery of **8a** which potently inhibited *h*DDAH-1 (K_i = 18 µM) without significantly affecting NOSs and arginase. Its guanidine enabled a unique binding mode that obviates the need for the α-carboxy group, which was not the case for corresponding amidine analogs. Importantly, these features opened an opportunity for designing a prodrug candidate of **8a**, i.e. *N*-hydroxyguanidine **11**, that eventually complemented a unique toolset of chemical modalities for experimental pharmacology with lead-like qualities for further development. Future directions should be devoted to further optimization of **8a** as a lead with the aim to improve potency towards the low µM range. Our structure-based data suggests that conformational restraints in the butyl chain might add to binding affinity, potentially regaining potency (and selectivity) for amidine-based inhibitors. Moreover, the acylsulfonamide-site of interaction postulated for ZST316 could represent an attractive option for further improvements despite the rather tight ligand binding pocket of DDAH.

EXPERIMENTAL SECTION

All final test compounds were purified to >95% as determined by combustion analysis or LC/MS. The analytical methods, general chemistry, experimental information, and syntheses of all other compounds are provided in the Supporting Information.

General procedure for the synthesis of guanidine derivatives (8). Protected carbamoylguanidines were prepared according to previously described protocols.[22, 23] Briefly, 1.5 eq DIPEA, the respective amine (e.g., **7a**) and EDCI were reacted with 0.5 mmol thiourea (e.g., **6a**) in 10 ml of dry CH_2Cl_2. Unless noted otherwise, reactions were complete after stirring overnight. The organic phase was diluted with 10 ml of CH_2Cl_2 and washed with small amounts of 1% aqueous HCl, water and brine. The resulting oils were purified by column chromatography on silica gel. For deprotection, the intermediates (e.g., **S3a**) were stirred in 10 ml of TFA and 3 ml thioanisole overnight. TFA was evaporated, 5 ml water, 15 ml Et_2O added, the organic phase extracted (2x) with 5 ml water and the combined aqueous phases washed once with Et_2O. The aqueous phase was concentrated and the crude products purified by chromatography.

***N*-(4-Aminobutyl)-*N*'-(2-methoxyethyl)guanidine bis(trifluoroacetate) (8a).** Purification by flash chromatography on RP-18 silica gel; eluent: 0.1 % TFA in water; ninhydrine positive fractions were pooled and lyophilized. Yield: 234 mg of a colourless oil (99%); R_f = 0.22 (*i*-PrOH/H_2O/AcOH, 8:2+0.5); ^{1}H NMR (DMSO-d_6): δ/ppm = 1.54 (m, 4H, N-CH_2-C$\underline{H_2}$-C$\underline{H_2}$), 2.80 (m, 2H, N-CH_2), 3.14 (m, 2H, N-CH_2), 3.27 (s, 3H, O-CH_3), 3.32, 3.42 (2 × t, 4H, N-CH_2-CH_2-O), 7.47 (br s, 2H, NH_2), 7.61 (br t, 1H, NH), 7.71 (br t, 1H, NH), 7.87 (br s, 3H, NH_3^+); ^{13}C NMR (DMSO-d_6): δ/ppm = 24.1 ($\underline{C}H_2$-CH_2-NH_3^+), 25.4 ($\underline{C}H_2$-CH_2-CH_2-NH_3^+), 38.3 (N-CH_2), 40.3 (N-CH_2), 40.7 (N-CH_2), 58.0 (O-CH_3), 70.0 (O-CH_2), 155.9 (C=N); MS (ESI): m/z = 189 $[M + H]^+$; Anal. calcd for $C_8H_{20}N_4O \cdot 2.5\ CF_3COOH$ (473.33): C 32.99, H 4.79, N 11.84; C 32.86, H 4.80, N 11.50.

***N*-Hydroxy-*N*´-(4-aminobutyl)-*N*´´-(2-methoxyethyl)guanidine Dihydrosulphate (11).** 400 mg (1 mmol) of the protected precursor (**S4**) were dissolved in 15 ml of a mixture from water and dioxane (2:1). 3 ml H_2SO_4 96% were added and the solution was stirred at room temperature for 3 hours. The pH was adjusted to 6 with $NaHCO_3$ and the mixture was dried in vacuum. The residue was dissolved in methanol and inorganic salts were removed by filtration. The mixture was dried in vacuum and the residue further purified by RP_{18} silica gel flash chromatography and eluted with Aqua bidest. Yield: 277 mg, colourless foam (89%). R_f = 0.46 (*i*-PrOH/H_2O/AcOH, 6:3:1); ^{1}H NMR (D_2O): δ/ppm = 1.74 (m, 4H, 2´,3´-CH_2), 3.06 (t, ^3J = 6.7 Hz, 2H, 4´-CH_2), 3.30 (t, ^3J = 6.1 Hz, 2H, 1´-CH_2), 3.42 (s, 3H, O-CH_3), 3.46 (t, ^3J = 5.0 Hz, 2H, 1´´-CH_2), 3.66 (t, 3J = 5.0 Hz, 2H, 2´´-CH2); 13C-NMR (D2O): δ/ppm = 24.0 (3´-CH_2), 25.0 (2´-CH_2), 39.0 (4´-CH_2), 40.3 (1´-CH_2), 40.8 (1´´-CH_2), 58.3 (O-CH_3), 70.3 (2´´-CH_2), 157.6 (C=N); ; MS (ESI): m/z = 205 [M + H]+. Anal. calcd for $C_8H_{22}N_4O_6S \cdot 1.0\ H_2O$ (320.37): C 29.99, H 7.55, N 17.49, S 10.01; found: C 29.99, H 7.89, N 17.12, S 10.64.

General procedure for the synthesis of amidine derivatives (16). Synthesis of amidine derivates (**16**) started with imidates (e.g., **14b**) as building blocks which were were prepared from the appropriate nitrile (e.g., **S8**) according to the literature with minor changes.[24] Briefly, imidates (e.g., **14b**) were added to 100 ml of cold, dry Et_2O to give a precipitate which has been (in most cases) collected by filtration, dried *in vacuo* (phosphorous pentoxide) and stored under argon at -20 °C. All imidates were white solids with yields between 90 % and 95 %. Two methods were employed for the preparation of the protected amidine precursors (e.g., *S10c*). Method B was used for the synthesis of amidine **16c**: N^{α}-Boc-, *O*-*t*Bu-protected L-ornithine (**15a**) (1 mmol) was dissolved in 15 ml MeOH at 0 °C and the imidate **14b** (3 mmol), DIPEA (1 ml) and DMAP (1 crystal) added. The mixture was stirred for 8 h at 0 °C and then overnight at room temperature. The solvent was evaporated and the products purified by column chromatography (SiO2, EtOAc/MeOH, 6:1). For deprotection, amidines (0.5 mmol) were dissolved in 5 ml TFA/CH_2Cl_2 (1:1) and stirred for 30 min at room temperature. The mixture was concentrated *in vacuo*, diluted with 5 ml water and washed twice with Et_2O. The aqueous phase was evaporated and the compounds further purified by flash column chromatography (RP18) with water as the eluent.

***N*^{5}-(1-Imino-3-methoxypropyl)-ornithine bis(trifluoroacetate) (16c).** Yield: 134 mg colourless oil (67%). R_f = 0.10 (*i*-PrOH/H_2O/AcOH, 8:2+0.5); ^{1}H-NMR (DMSO-*d*6): δ/ppm = 1.53-1.90 (m, 4H, 3,4-CH_2), 2.65 (t, ^3J = 6.1 Hz, 2H, CH_2-C=N), 3.23 (t, ^3J = 6.2 Hz, 2H, 5-CH_2), 3.25 (s, 3H, CH_3-O), 3.64 (t, ^3J = 6.1 Hz, 2H, O-CH_2), 3.80 (t, ^3J = 6.2 Hz, 1H, CH), 8.80, 9.31, 9.77 (3 x br s, 1H, NH); ^{13}C-NMR (DMSO-d6): δ/ppm = 23.1 (4-CH_2), 27.2 (3-CH_2), 33.0 (CH_2-C=N), 40.9 (5-CH_2), 51.6 (CH), 58.9 (CH_3), 67.9 (O-CH_2), 164.0 (C=O^{t}Bu),

170.8 (C=N); MS (ESI): m/z = 218 $[M + H]^+$; Anal. calcd for $C_9H_{19}N_3O_3 \cdot 1.5\ CF_3COOH \cdot 0.8\ H_2O$ (402.72): C 35.79, H 5.53, N 10.43, found: C 35.76, H 5.38, N 10.54.

Biochemical assays for *h*DDAH-1, NOSs and arginase. Assays were performed according to our described procedures and can be found in the Supporting Information file.[14, 21]

***In vitro* bioactivation.** Incubations of prodrug **11** with distinct subcellular porcine liver enzyme sources and the reconstituted human mARC-1 and -2 systems were performed as previously described.[31] Guanidine **8a** was quantified by HPLC using a precolumn derivatization protocol (*o*-phthalaldehyde).

***h*DDAH-1 crystal structures and binding mode analysis.** Protein expression and purification of *h*DDAH-1 was done as described previously.[14] Details on crystallization and structure solution can be found in the Supporting Information file. Atomic coordinates are published in the RCSB Protein Data Bank (apo-*h*DDAH-1 pdb: 6szq, *h*DDAH-1 **8a** complex pdb: 6szp).

ASSOCIATED CONTENT

Supporting Information. Synthesis and characterization of all intermediates and final compounds; Description of biochemical assays for *h*DDAH-, NOS- and arginase-inhibition, and bioactivation studies; HPLC method for the quantification of **8a** and **11** (Fig. S1-S2); stability assessment of **11** (Fig. S3); X-ray data collection and refinement statistics (Table S1); *h*DDAH-1 crystal structures and augmented reality sessions (Fig. S4-S7). Molecular Formula Strings are available. This material is available free of charge via the Internet at http://pubs.acs.org.

AUTHOR INFORMATION

Corresponding Author

*For D.S.: phone, +49 431 8801176; e-mail, schade@pharmazie.uni-kiel.de. ORCID-ID: 0000-0002-5515-1821.

Notes

The authors decare no competing financial interest.

Author Contributions

The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript. ‡These authors contributed equally.

ACKNOWLEDGMENT

We thank Dr. U. Girreser for analytical service and M. Zietz and S. Wichmann for excellent technical assistance. We acknowledge support by the beamline staff of the X10SA SLS (Paul Scherrer I., Villigen), the P11 at PETRA synchrotron (Hamburg), as well as our colleagues at MPI Dortmund for help with the data collection. We thank Dr. M. P. Müller for assistance with the AR-protocol.

ABBREVIATIONS

L-IPeO, N^5-(1-iminopentyl)-L-ornithine; L-VNIO, N^5-(1-imino-3-butenyl)-L-ornithine; mARC, mitochondrial amidoxime reducing component; NOS, nitric oxide synthase.

PDB ID CODES

pdb 2jaj (compound: **1**), pdb 3p8p (compound: **L-IPeO**), pdb 2jai (compound: **L-citrulline**), pdb 6szp (compound: **8a**), pdb 6szq (apo-*h*DDAH-1). Authors will release the atomic coordinates and experimental data upon article publication.

REFERENCES

1. Schade, D.; Kotthaus, J.; Clement, B., Modulating the NO generating system from a medicinal chemistry perspective: current trends and therapeutic options in cardiovascular disease. *Pharmacol. Ther.* **2010,** *126* (3), 279-300.

2. Leiper, J.; Nandi, M., The therapeutic potential of targeting endogenous inhibitors of nitric oxide synthesis. *Nat. Rev. Drug Discov.* **2011,** *10* (4), 277-291.

3. Leiper, J.; Nandi, M.; Torondel, B.; Murray-Rust, J.; Malaki, M.; O'Hara, B.; Rossiter, S.; Anthony, S.; Madhani, M.; Selwood, D.; Smith, C.; Wojciak-Stothard, B.; Rudiger, A.; Stidwill, R.; McDonald, N. Q.; Vallance, P., Disruption of methylarginine metabolism impairs vascular homeostasis. *Nat. Med.* **2007,** *13* (2), 198–203.

4. Pullamsetti, S. S.; Savai, R.; Dumitrascu, R.; Dahal, B. K.; Wilhelm, J.; Konigshoff, M.; Zakrzewicz, D.; Ghofrani, H. A.; Weissmann, N.; Eickelberg, O.; Guenther, A.; Leiper, J.; Seeger, W.; Grimminger, F.; Schermuly, R. T., The role of dimethylarginine dimethylaminohydrolase in idiopathic pulmonary fibrosis. *Sci. Transl. Med.* **2011,** *3* (87), 87ra53.

5. Wang, Y.; Hu, S.; Gabisi, A. M., Jr.; Er, J. A.; Pope, A.; Burstein, G.; Schardon, C. L.; Cardounel, A. J.; Ekmekcioglu, S.; Fast, W., Developing an irreversible inhibitor of human DDAH-1, an enzyme upregulated in melanoma. *ChemMedChem* **2014,** *9* (4), 792-797.

6. Kostourou, V.; Robinson, S. P.; Cartwright, J. E.; Whitley, G. S., Dimethylarginine dimethylaminohydrolase I enhances tumour growth and angiogenesis. *Br. J. Cancer* **2002,** *87* (6), 673-680.

7. Reddy, K. R. K.; Dasari, C.; Duscharla, D.; Supriya, B.; Ram, N. S.; Surekha, M. V.; Kumar, J. M.; Ummanni, R., Dimethylarginine dimethylaminohydrolase-1 (DDAH1) is frequently upregulated in prostate cancer, and its overexpression conveys tumor growth and angiogenesis by metabolizing asymmetric dimethylarginine (ADMA). *Angiogenesis* **2018,** *21* (1), 79-94.

8. Murphy, R. B.; Tommasi, S.; Lewis, B. C.; Mangoni, A. A., Inhibitors of the hydrolytic enzyme dimethylarginine dimethylaminohydrolase (DDAH): Discovery, synthesis and development. *Molecules* **2016,** *21* (5), pii: E615.

9. Stone, E. M.; Schaller, T. H.; Bianchi, H.; Person, M. D.; Fast, W., Inactivation of two diverse enzymes in the amidinotransferase superfamily by 2-chloroacetamidine: dimethylargininase and peptidylarginine deiminase. *Biochemistry* **2005,** *44* (42), 13744-13752.

10. Knipp, M.; Braun, O.; Vasak, M., Searching for DDAH inhibitors: S-nitroso-L-homocysteine is a chemical lead. *J. Am. Chem. Soc.* **2005,** *127* (8), 2372-2373.

11. Rossiter, S.; Smith, C. L.; Malaki, M.; Nandi, M.; Gill, H.; Leiper, J. M.; Vallance, P.; Selwood, D. L., Selective substrate-based inhibitors of mammalian dimethylarginine dimethylaminohydrolase. *J. Med. Chem.* **2005,** *48* (14), 4670–4678.

12. Tommasi, S.; Zanato, C.; Lewis, B. C.; Nair, P. C.; Dall'Angelo, S.; Zanda, M.; Mangoni, A. A., Arginine analogues incorporating carboxylate bioisosteric functions are micromolar inhibitors of human recombinant DDAH-1. *Org. Biomol. Chem.* **2015,** *13* (46), 11315–11330.

13. Hulin, J.-A.; Tommasi, S.; Elliot, D.; Mangoni, A. A., Small molecule inhibition of DDAH1 significantly attenuates triple negative breast cancer cell vasculogenic mimicry in vitro. *Biomed. Pharmacother.* **2019,** *111*, 602–612.

14. Kotthaus, J.; Schade, D.; Muschick, N.; Beitz, E.; Clement, B., Structure-activity relationship of novel and known inhibitors of human dimethylarginine dimethylaminohydrolase-1: Alkenyl-amidines as new leads. *Bioorg. Med. Chem.* **2008,** *16* (24), 10205–10209.

15. Wang, Y.; Monzingo, A. F.; Hu, S.; Schaller, T. H.; Robertus, J. D.; Fast, W., Developing dual and specific inhibitors of dimethylarginine dimethylaminohydrolase-1 and nitric oxide synthase: Toward a targeted polypharmacology to control nitric oxide. *Biochemistry* **2009,** *48* (36), 8624–8635.

16. Wang, Y.; Hu, S.; Gabisi, A. M.; Er, J. A. V.; Pope, A.; Burstein, G.; Schardon, C. L.; Cardounel, A. J.; Ekmekcioglu, S.; Fast, W., Developing an irreversible inhibitor of human DDAH-1, an

enzyme upregulated in melanoma. *ChemMedChem* **2014,** *9* (4), 792–797.

17. Burstein-Teitelbaum, G.; Er, J. A. V.; Monzingo, A. F.; Tuley, A.; Fast, W., Dissection, optimization, and structural analysis of a covalent irreversible DDAH1 inhibitor. *Biochemistry* **2018,** *57* (30), 4574–4582.

18. Johnson, C. M.; Monzingo, A. F.; Ke, Z.; Yoon, D. W.; Linsky, T. W.; Guo, H.; Robertus, J. D.; Fast, W., On the mechanism of dimethylarginine dimethylaminohydrolase inactivation by 4-halopyridines. *J. Am. Chem. Soc.* **2011,** *133* (28), 10951-10959.

19. Linsky, T.; Wang, Y.; Fast, W., Screening for dimethylarginine dimethylaminohydrolase inhibitors reveals ebselen as a bioavailable inactivator. *ACS Med. Chem. Lett.* **2011,** *2* (8), 592-596.

20. Ghebremariam, Y. T.; Erlanson, D. A.; Cooke, J. P., A novel and potent inhibitor of dimethylarginine dimethylaminohydrolase: A modulator of cardiovascular nitric oxide. *J. Pharmacol. Exp. Ther.* **2014,** *348* (1), 69–76.

21. Kotthaus, J.; Schade, D.; Kotthaus, J.; Clement, B., Designing modulators of dimethylarginine dimethylaminohydrolase (DDAH): A focus on selectivity over arginase. *J. Enzyme Inh. Med. Chem.* **2012,** *27* (1), 24–28.

22. Martin, N. I.; Woodward, J. J.; Marletta, M. A., NG-hydroxyguanidines from primary amines. *Org. Lett.* **2006,** *8* (18), 4035–4038.

23. Schade, D.; Kotthaus, J.; Clement, B., Efficient synthesis of optically pure Nω-alkylated l-arginines. *Synthesis* **2008,** *2008* (15), 2391–2397.

24. Bretscher, L. E.; Li, H.; Poulos, T. L.; Griffith, O. W., Structural characterization and kinetics of nitric-oxide synthase inhibition by novel N5-(iminoalkyl)- and N5-(iminoalkenyl)-ornithines. *J. Biol. Chem.* **2003,** *278* (47), 46789–46797.

25. Babu, B. R.; Griffith, O. W., N5-(1-Imino-3-butenyl)-L-ornithine. A neuronal isoform selective mechanism-based inactivator of nitric oxide synthase. *J. Biol. Chem.* **1998,** *273* (15), 8882–8889.

26. Lluis, M.; Wang, Y.; Monzingo, A. F.; Fast, W.; Robertus, J. D., Characterization of C-alkyl amidines as bioavailable covalent reversible inhibitors of human DDAH-1. *ChemMedChem* **2011,** *6* (1), 81-88.

27. Ott, G.; Havemeyer, A.; Clement, B., The mammalian molybdenum enzymes of mARC. *J. Biol. Inorg. Chem.* **2015,** *20* (2), 265–275.

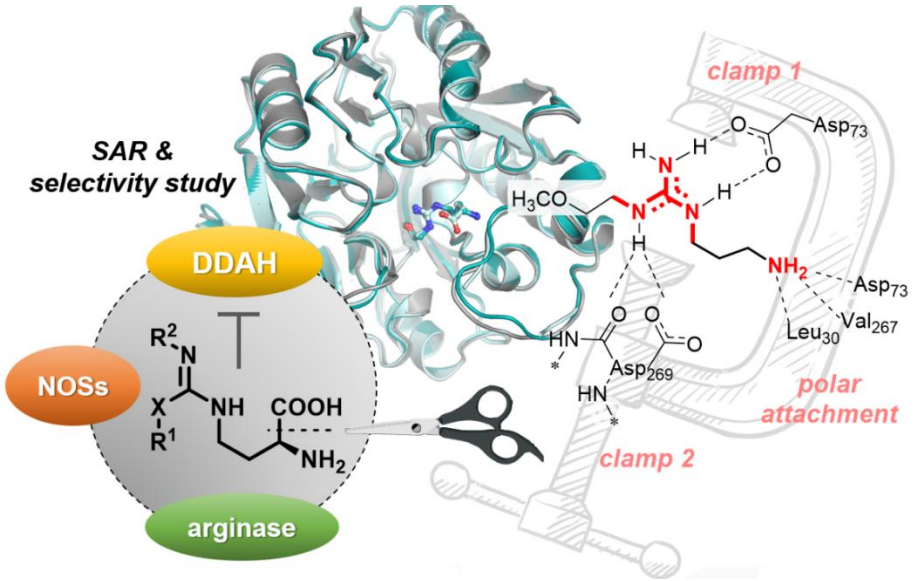
28. Plitzko, B.; Ott, G.; Reichmann, D.; Henderson, C. J.; Wolf, C. R.; Mendel, R.; Bittner, F.; Clement, B.; Havemeyer, A., The involvement of mitochondrial amidoxime reducing components 1 and 2 and mitochondrial cytochrome b5 in N-reductive metabolism in human cells. *J. Biol. Chem.* **2013,** *288* (28), 20228–20237.

29. Ott, G.; Plitzko, B.; Krischkowski, C.; Reichmann, D.; Bittner, F.; Mendel, R. R.; Kunze, T.; Clement, B.; Havemeyer, A., Reduction of sulfamethoxazole hydroxylamine (SMX-HA) by the mitochondrial amidoxime reducing component (mARC). *Chem. Res. Toxicol.* **2014,** *27* (10), 1687–1695.

30. Froriep, D.; Clement, B.; Bittner, F.; Mendel, R. R.; Reichmann, D.; Schmalix, W.; Havemeyer, A., Activation of the anti-cancer agent upamostat by the mARC enzyme system. *Xenobiotica* **2013,** *43* (9), 780–784.

31. Wahl, B.; Reichmann, D.; Niks, D.; Krompholz, N.; Havemeyer, A.; Clement, B.; Messerschmidt, T.; Rothkegel, M.; Biester, H.; Hille, R.; Mendel, R. R.; Bittner, F., Biochemical and spectroscopic characterization of the human mitochondrial amidoxime reducing components hmARC-1 and hmARC-2 suggests the existence of a new molybdenum enzyme family in eukaryotes. *J. Biol. Chem.* **2010,** *285* (48), 37847–37859.

Table of Contents Graphic



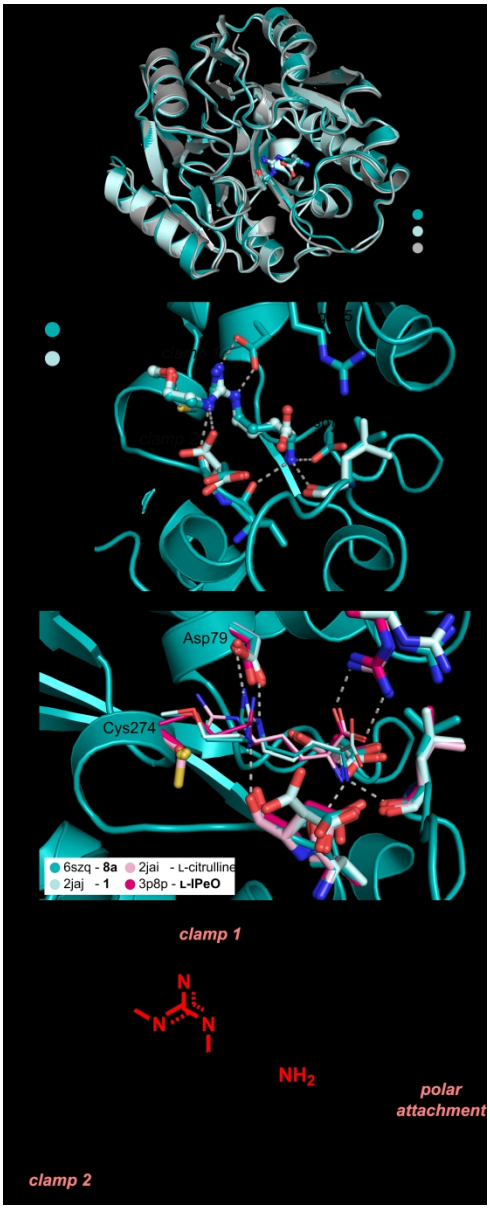


Figure 2. Binding mode analysis of guanidine- and amidine-types of *h*DDAH-1 inhibitors. **A)** Superposition of crys-tal structures of ligand-free (apo) or **1**- or **8a**-bound *h*DDAH-1; **B)** Binding modes of superimposed **8a** and **1**, dashed lines indicate interacting residues; C) 8a-bound *h*DDAH-1 with superimposed 1 (= guanidines), L-citrulline (= urea) and **L-IPeO** (= amidine). **D)** Schematic view on **8a** binding mode, illustrating contributions by active site amino acids.

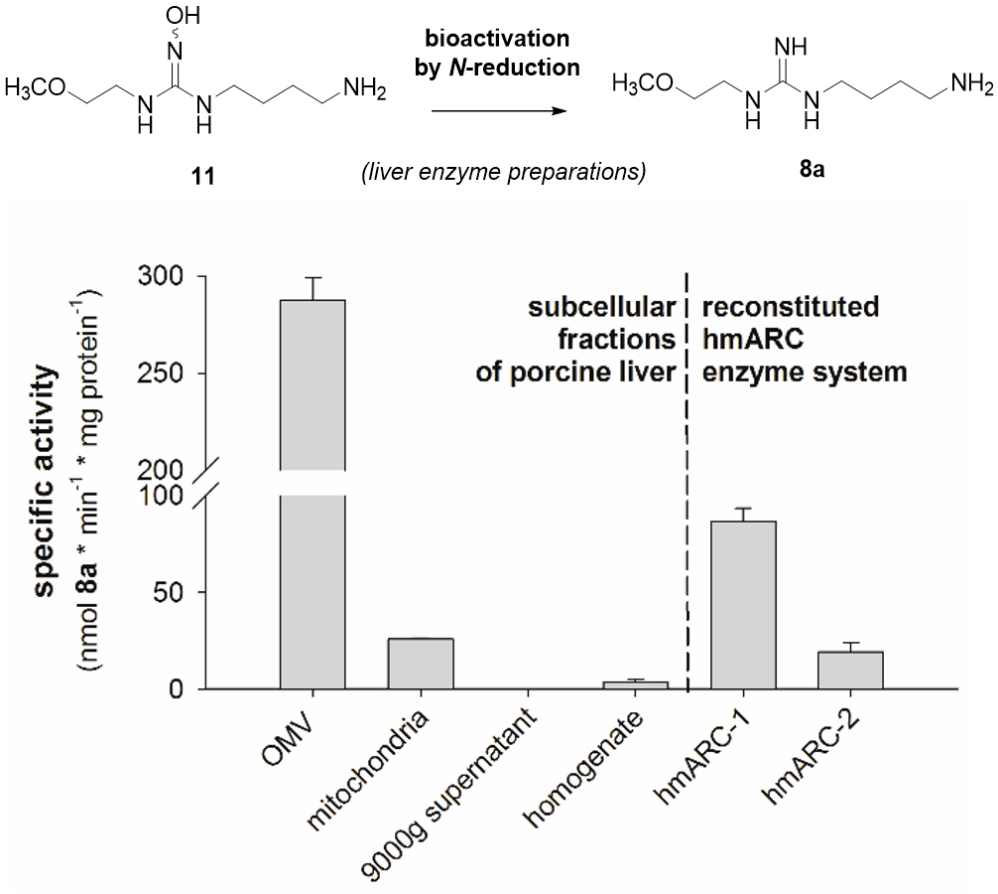
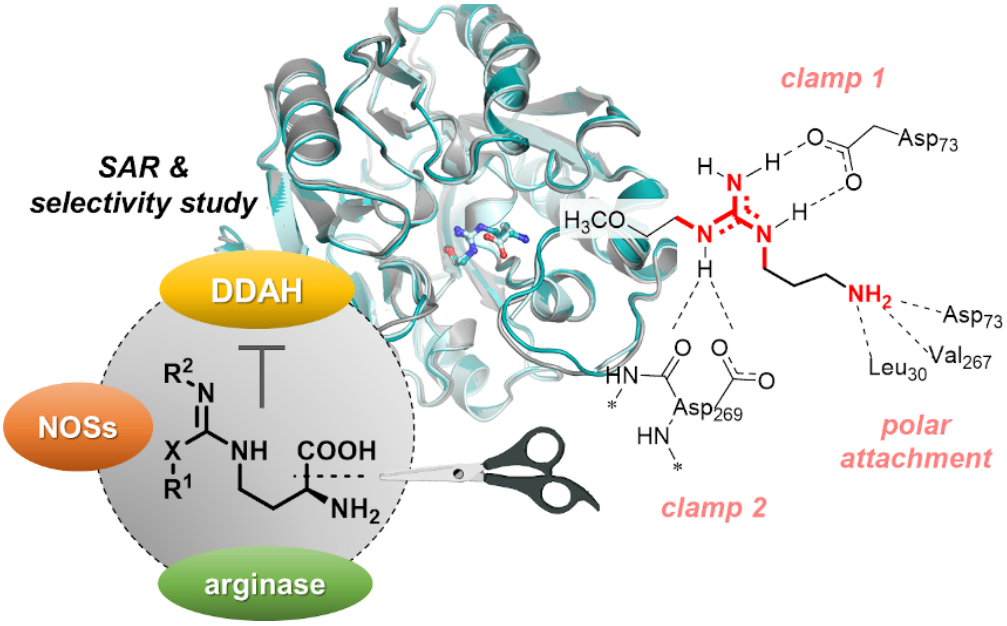


Figure 3. *In vitro* bioactivation of *N*-hydroxyguanidine **11** to **8a**.[a]

170x153mm (150 x 150 DPI)



TOC Graphic

72x44mm (300 x 300 DPI)