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RESEARCH ARTICLE

Designing modulators of dimethylarginine dimethylaminohydrolase (DDAH): A focus on selectivity over arginase

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Abstract

DDAH inhibition presents a novel promising pharmaceutical strategy to lower NO formation. To date, several potent DDAH inhibitors have been published, most of them representing analogues of L-arginine. While inhibitory effects on NOSs have already been considered, selectivity over arginase has been neglected so far. In our view, the latter selectivity is more important since an additional inhibition of arginase decreases the desired effects on NO levels. Thus, we particularly focus on selectivity over arginase. We present a comprehensive selectivity profile of known DDAH inhibitors by covering their inhibitory potency on arginase. Among the studied compounds, N^{ω}-(2-methoxyethyl)-L-arginine (**2a**, L-257) that is already selective over NOSs also only modestly affected arginase activity and is thus far the most suitable DDAH inhibitor for pharmacological studies.

Keywords: Nitric oxide, nitric oxide synthase, L-257, arginase, dimethylarginine dimethylaminohydrolase

Introduction

Nitric oxide (NO) is formed via nitric oxide synthases (NOSs) 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)[1]. The major physiological route of NMMA and ADMA degradation is via dimethylarginine dimethylaminohydrolase (DDAH) to L-citrulline and either dimethylamine or methylamine (see Figure 1)[2]. To date, two different isoforms have been described: DDAH-1 is mainly expressed in liver and kidney, whereas DDAH-2 is located in the vascular endothelium, heart, placenta and kidney[3]. Thus, there is a colocalization of DDAH-1/nNOS and DDAH-2/eNOS, indicating an isoform-specific regulation of NOS activity[4].

Accordingly, DDAH inhibition represents a promising pharmaceutical strategy to indirectly affect NO formation by elevating N^{ω}-methylated L-arginine levels[5]. This hypothesis was supported by former studies showing that pharmacological inhibition of DDAH results in an accumulation of ADMA and subsequently decreased NO formation[6,7]. Thus, DDAH inhibition might be useful for the therapy of pathophysiological conditions associated with NO overproduction such as cardiogenic shock, pain, migraine, neurodegenerative diseases (Alzheimer's disease), or it might be a pharmaceutical strategy to reduce angiogenesis associated with tumours, arthritis and diabetic retinopathy[8,9].

At present, several L-arginine and N^{5}-(1-imino-alk(en)yl)-L-ornithine derivatives have been identified as DDAH inhibitors, with N^{5}-(1-iminobut-3-enyl)-L-ornithine (**1a**) and N^{ω}-(2-methoxyethyl)-L-arginine (**2a**, L-257) as the most potent representatives. However, most of these L-arginine analogues additionally affect NOSs activity[7,10–12]. In fact, it is still a matter of debate whether selectivity over NOSs is essentially needed, considering that inhibition of both NOSs and DDAH represents a dual mode of action and should increase potency of

Address for Correspondence: Dr. Bernd Clement, Pharmaceutical and Medicinal Chemistry, Christian-Albrechts-University, Gutenbergstr. 76-78, Kiel, 24118 Germany. Tel: +49-431-8801126. Fax: +49-431-8801352. E-mail: bclement@pharmazie.uni-kiel.de

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Abbreviations

ADMA, asymmetric N^{ω},N^{ω}-dimethyl-L-arginine
DDAH, dimethylarginine dimethylaminohydrolase
NMMA, N^{ω}-monomethyl-L-arginine
NO, nitric oxide
NOS, nitric oxide synthase
SARs, structure activity relationships

these compounds[9,11]. However, taken into account that all direct NOS inhibitors failed in clinical trials, we think that selectivity over NOSs is desirable or at least inhibitory effects on NOS should be minimized. Further studies (preferably *in vivo*) are needed to show whether or not a highly specific DDAH inhibitor is superior over a dual DDAH/NOS inhibitor.

With regards to enzyme selectivity most published works dealing with DDAH inhibitors merely considered selectivity over NOS isoforms, whereas selectivity over arginase has never been investigated at all. However, arginases and NOSs are the predominant enzymes in L-arginine metabolism and compete for their common substrate L-arginine (see Figure 1)[13]. Hence, arginase inhibition elevates NO formation by augmenting the L-arginine substrate pool for NOSs. Meanwhile, several studies confirmed this reciprocal interaction between both pathways under physiological conditions[9,14,15]. Additionally, taken the different K_m-values for NOS (~5 μM) and arginase (~5 mM) for L-arginine into account, inhibitors showing comparable K_i-values for these enzymes can be assumed to mainly affect arginase activity[15,16]. Thus, we assume that selectivity over arginase is even more important than selectivity over NOSs, since an additional inhibition of arginase would lower the desired pharmacological effect of DDAH inhibition[9]. In the presented work, we addressed this selectivity issue for a selection of DDAH inhibitors as this has been so far neglected in the development of such pharmacological tools or drug candidates.

Methods

General

Commercially available materials were purchased from either Sigma-Aldrich (Seelze, Germany), Fluka (Buchs, Switzerland), Merck (Darmstadt, Germany), Alexis Biochemicals (Lausen, Switzerland) or Roth (Karlsruhe, Germany) unless otherwise stated. NMMA, **1a**, **2b** were purchased from Alexis Biochemicals. **1d** and **1e** were purchased from Cayman chemicals (Ann Arbor, Michigan, USA). N^{ω}-substituted L-arginine derivatives except for **2b** and **2g** were synthesized according to Schade et al.[17]. **1b** and **1c** were obtained from BogaR Laboratories LLC (Alpharetta, Georgia, USA).

Expression and purification of 6xHis-tagged hDDAH-1

Human DDAH-1 was expressed in *Escherichia coli* BL21 and purified via Ni-NTA-agarose as described previously[18].

In vitro hDDAH-1 assay

A colorimetric assay was carried out in 150 μL 50 mM potassium phosphate buffer pH 7.4 containing 4 μg of recombinant hDDAH-1 and varying concentrations of NMMA and inhibitor. NMMA was applied at 50, 100, 300, 700 and 1250 μM. Each dilution was incubated each inhibitor in five different concentrations and one without inhibitor as a control. 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[19]. Microplates were sealed with sealing tape, incubated at 95°C for 20 min and then read at 540.5 nm. Subsequent, K_i-values were calculated using SigmaPlot 8.0 (SPSS Inc.) and Microsoft Excel.

In vitro NOS assay

Inhibition studies were carried out as described previously[10].

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 100 μM maleic acid and 7 mM L-arginine. Inhibitors were applied at concentrations of 100 μM and 1 mM. 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[19]. Microplates were sealed with sealing tape, incubated at 95°C for 20 min and then read at 526 nm.

Results

In vitro DDAH-assay

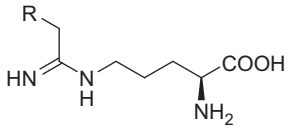
Initially, we investigated biochemical properties of our recombinantly expressed hDDAH-1 and determined the K_m-value for NMMA (133 ± 75 μM) and V_{max} (210 ± 14 nmol × min^{-1} × mg^{-1}), which lies in the same range as published values of other groups[16].

We previously reported of several novel DDAH-1 inhibitors[10]. In this work, we determined K_i-values for all these compounds for hDDAH-1. **1a**, **1b**, **1c**, **2a**, and **2e** showed highest potency (Table 1).

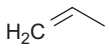
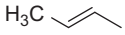
In vitro arginase assay

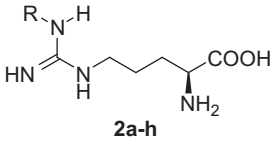
We established an easy plate reader assay format to test the effects on arginase. Our study revealed that most potent DDAH-1 inhibitors additionally inhibit arginase activity (Table 1). In particular, the most potent DDAH-1 inhibitor **1a** known so far, turned out to be a moderately potent inhibitor of arginase, as well. Other compounds moderately affecting arginase activity are **2b** and **2d**.

Table 1. Inhibition data of N^5-(1-imino-alk(en)yl)-L-ornithine (**1**) and N^{ω}-substituted L-arginine (**2**) derivatives. NOS and DDAH inhibition data is taken from a former publication[10]. Data stated in brackets are inhibition data at 1 mM.

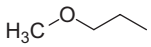
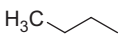
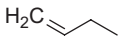
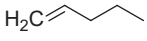
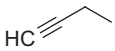
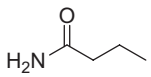


1a-e

Compound	*R*	K_i (μM) hDDAH-1	Inhibition at 100 μM (%) nNOS	eNOS	iNOS	Arginase
1a		2 ± 1	94 ± 3 (100 ± 1)	45 ± 2 (88 ± 3)	84 ± 1 (100 ± 1)	25 ± 9 (76 ± 10)
1b		32 ± 6	34 ± 8 (100 ± 1)	20 ± 6 (54 ± 3)	80 ± 3 (100 ± 1)	21 ± 9 (30 ± 10)
1c		36 ± 3	12 ± 11 (70 ± 8)	27 ± 8 (36 ± 1)	6 ± 2 (21 ± 11)	8 ± 3 (12 ± 7)
1d		145 ± 15	77 ± 2 (100 ± 1)	20 ± 3 (85 ± 6)	72 ± 2 (100 ± 1)	26 ± 3 (30 ± 5)
1e	H	1155 ± 112	75 ± 4 (100 ± 1)	80 ± 5 (100 ± 1)	84 ± 3 (100 ± 1)	20 ± 5 (31 ± 8)



2a-h

Compound	*R*	K_i (μM) hDDAH-1	Inhibition at 100 μM (%) nNOS	eNOS	iNOS	Arginase
2a		13 ± 2	4 ± 12 (12 ± 8)	4 ± 7 (10 ± 5)	0 ± 13 (0 ± 13)	16 ± 7 (29 ± 6)
2b		90 ± 5	68 ± 3 (100 ± 1)	15 ± 10 (55 ± 6)	0 ± 3 (2 ± 15)	20 ± 10 (74 ± 11)
2c		58 ± 9	68 ± 2 (100 ± 1)	21 ± 5 (75 ± 5)	30 ± 8 (100 ± 1)	13 ± 7 (33 ± 11)
2d		57 ± 9	0 ± 11 (14 ± 4)	16 ± 5 (35 ± 5)	0 ± 3 (40 ± 8)	31 ± 12 (70 ± 21)
2e		17 ± 5	71 ± 3 (100 ± 1)	20 ± 4 (87 ± 2)	58 ± 4 (100 ± 1)	20 ± 8 (27 ± 10)
2f		606 ± 66	17 ± 4 (34 ± 4)	15 ± 1 (30 ± 6)	0 ± 9 (23 ± 6)	11 ± 4 (27 ± 1)
2g		1968 ± 290	100 ± 1 (100 ± 1)	100 ± 1 (100 ± 1)	91 ± 8 (100 ± 1)	14 ± 8 (3 ± 6)
2h		764 ± 107	11 ± 5 (14 ± 4)	15 ± 10 (37 ± 6)	0 ± 7 (30 ± 6)	18 ± 3 (27 ± 8)

DDAH, dimethylarginine dimethylaminohydrolase; NOS, nitric oxide synthases.

Compound **2a** (L-257) turned out to be not only selective over NOS but also over arginase. Regarding compounds of series **1** it is observable that **1c** has minimal side effects on arginase activity.

Discussion

We previously reported several novel human DDAH inhibitors of which **1a**, **1b**, **1c**, **2a**, and **2e** showed highest potency. Results of the herein presented arginase assay revealed that most potent DDAH inhibitors additionally inhibit arginase activity. In particular, the most potent DDAH inhibitor known so far **1a**, turned out to be a moderately potent inhibitor of arginase as well, limiting its applicability in further studies. In contrast, **1c** has minimal side effects on arginase activity, whereas it is not entirely selective over NOSs and about 2.5-fold less potent than **2a** (L-257). Nevertheless, this compound seems to be suitable for further studies. Interestingly, **1b** which differs from **1a** in a double-bond exhibits less effects on arginase activity. Within series **2,** compound **2a** (L-257) turned out to be not only exceptionally selective over NOS but also over arginase. Additionally, **2c** and **2e** represent potent DDAH inhibitors possessing a moderate selectivity over arginase; however, as for **1b** these compounds are not selective over NOSs. Nevertheless, these compounds might represent interesting model compounds since other authors claim the dual inhibition of NOSs and DDAH as a promising pharmaceutical strategy[11]. **2b** and **2d** are other compounds that slightly more potently affected arginase activity limiting their applicability in further studies. In particular, **2d** originally was deemed to be a promising lead for further studies because of its high selectivity over NOSs.

The most potent arginase inhibitors known so far (i.e. the boronic acid and *N*-hydroxylated L-arginine derivatives) mimic the tetrahedral intermediate of L-arginine in arginase catalysis[20,21]. In contrast, other L-arginine derivatives turned out to be much less effective. However, at present our knowledge about structure

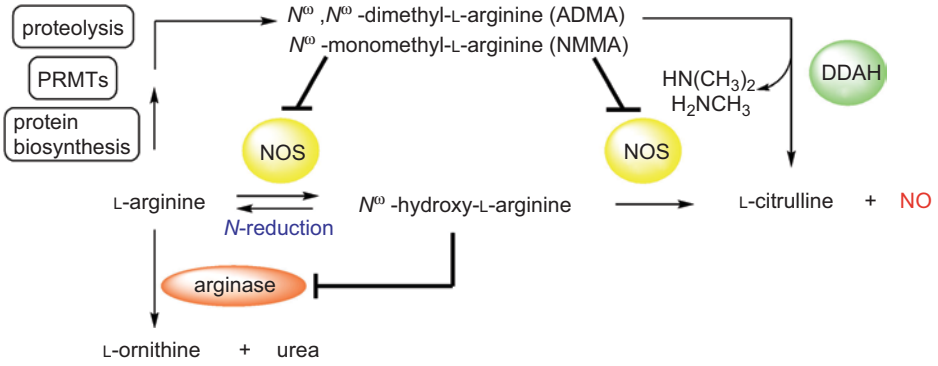


Figure 1. Overview on the NO generating system and its physiological regulation. N-reduction, microsomal/mitochondrial reduction23. DDAH, dimethylarginine dimethylaminohydrolase; NOS, nitric oxide synthase; PRMT, protein arginine methyltransferase.

activity relationships (SARs) of moderately potent arginase inhibitors such as **1a**, **2b**, and **2d** is still very restricted and we cannot easily predict their inhibitory potency, but rather have to rely on empirical knowledge based on our *in vitro* selectivity profile studies. Nevertheless, with the herein presented data we tried to further deduce SARs for DDAH inhibitors under consideration of arginase cross-inhibition, but:

1. In respect of the N^{ω}- (L-arginines/guanidines) or N^{5}- (amidines) substituent length, compounds showing greatest side effects on arginase are **1a**, **2b**, and **2d** which all differ in substituent length.
2. When comparing some alkyl derivatives with their corresponding alkenyl derivatives for compounds **1a**/**1b** the alkenyl derivative more potently inhibits arginase. However, for compound pair **2b**/**2c** the alkyl derivative is much less selective over arginase. Thus, regarding the influence of multiple bonds no general conclusions can be drawn. Hence, at this point no general conclusions regarding SARs for the development of arginase selective DDAH inhibitors can be drawn, underlining the necessity of investigating arginase selectivity for each newly developed DDAH inhibitor.

Conclusions

In summary, the present study underlines the importance to more enlighten the influence of potential DDAH inhibitors on arginase activity, aside from other enzymes involved in NO-related metabolism. Thus, investigating selectivity over arginase should be performed as a standard assay in all further studies dealing with the development of DDAH inhibitors.

Essentially, **2a** (L-257) developed by the Leiper group represents the most promising DDAH inhibitor known so far due to its good selectivity profile over the other L-arginine converting enzymes[7,8]. This compound turned out to be not only selective over NOSs—as already known—but also over arginase. Although, we think that DDAH inhibitors should be selective for this enzyme, other authors claim a dual DDAH/NOS inhibition as promising[11]. Based on this aspect, **1c**, **2c**, and **2e** may also be useful in future studies.

The herein presented study reveals the necessity of addressing arginase selectivity issues, since **1a**, the most potent DDAH inhibitor known so far, and **2d** also effectively inhibit arginase, and thus, despite their potency on DDAH cannot be considered as suitable candidates for studies that are aimed at investigating DDAH specific (patho)physiological effects. Nevertheless, our knowledge about the broad spectrum of DDAH (patho) physiological functions is still rather low at present[9]. In particular, more studies are needed to investigate the *in vivo* effects of an additional arginase inhibition in order to support or possibly disprove our assumption that selectivity over arginase is more important than selectivity over NOS. Additionally, isoform selectivity of DDAH inhibitors requires further investigations. But recombinant expression of DDAH-2 is a challenging task, and to date, no working group succeeded in recombinant expression and isolation of this isoform. Therefore, current inhibition data is exclusively available for DDAH-1. However, due to the different tissue distribution isoform-specific inhibitors bear the potential to affect DDAH activity—and consequently NOS activity—in specific tissues[22].

Declaration of interest

The authors report no conflicts of interest.

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