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Heteroalicyclic carboxamidines as inhibitors of inducible nitric oxide synthase; the identification of (2*R*)-2-pyrrolidinecarboxamidine as a potent and selective haem-co-ordinating inhibitor

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ABSTRACT

Heteroalicyclic carboxamidines were synthesised and evaluated as inhibitors of nitric oxide synthases. (2*R*)-2-Pyrrolidinecarboxamidine, in particular, was shown to be a highly potent in vitro (IC_{50} = 0.12 μM) and selective iNOS inhibitor (>100-fold vs both eNOS and nNOS), with probable binding to the key anchoring glutamate residue and co-ordination to the haem iron.

The simple radical gas nitric oxide (NO), which was shown to be the endothelium derived relaxing factor,[1] has been implicated in a diverse range of physiological roles.[2] It is produced by oxidation of L-arginine by one of three isoforms of Nitric Oxide Synthase (NOS).[3] Picomolar concentrations of NO have essential roles in vascular homeostasis and neurotransmission; produced by the constitutive, calcium-regulated, isoforms endothelial (eNOS) and neuronal (nNOS) respectively.[4] Nanomolar concentrations of NO are produced by the inducible isoform (iNOS), which is produced in response to stimuli such as cytokines or bacterial endotoxins, to play an essential role in host defence mechanisms.[5] However, overproduction of NO can have pathophysiological consequences; the upregulation of iNOS is implicated in septic shock[6] and a number of inflammatory conditions.[7] The selective inhibition of iNOS (in order to maintain the beneficial actions of the constitutive isoforms) has therefore been an attractive and well-pursued therapeutic target.[8] However, to date, few compounds with genuine potency and selectivity have been discovered;[9] amongst this class, the amino acid **GW274150**[10] has progressed furthest in clinical trials.[11]

The majority of potent inhibitors feature a basic amidine or guanidine motif, which satisfies the same binding interactions that recognise guanidine in the natural substrate, L-arginine (Fig. 1A). Specifically, there are three hydrogen-bonding interactions between two nitrogens of the guanidine and the conserved glutamate residue and a backbone tryptophan carbonyl.[12] The third nitrogen of the guanidine is thus held in close proximity to the haem iron to facilitate its oxidation to NO.[13,14] Evidence to date of compounds exploiting both the glutamate–amidine salt bridge and forming a classical type II co-ordination of the haem iron has been limited to nNOS inhibitors with thioether substituted actetamidine motifs.[15] In their detailed study, Martell et al. conclude that hydrophobic contacts around the haem facilitate a strong contribution to stabilisation of the sulphur–iron binding. Thiocitrulline was pur-

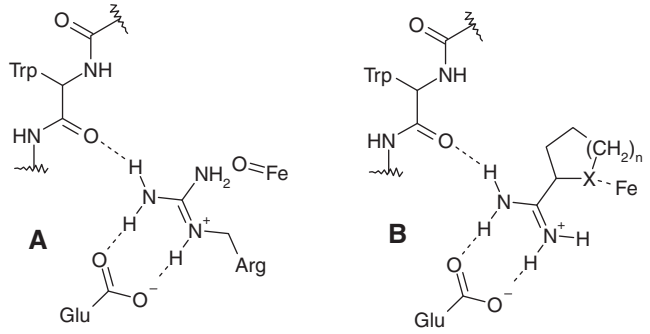

Figure 1. Binding pattern of conserved Glu and Trp residues,[12] which, in **A**, bind the guanidine motif of the arginine substrate above the haem iron to facilitate oxidation in all NOS isoforms. The envisaged heterocyclic carboxamidine interactions are shown in **B**, illustrating the backbone recognition and proximity of the haem iron.

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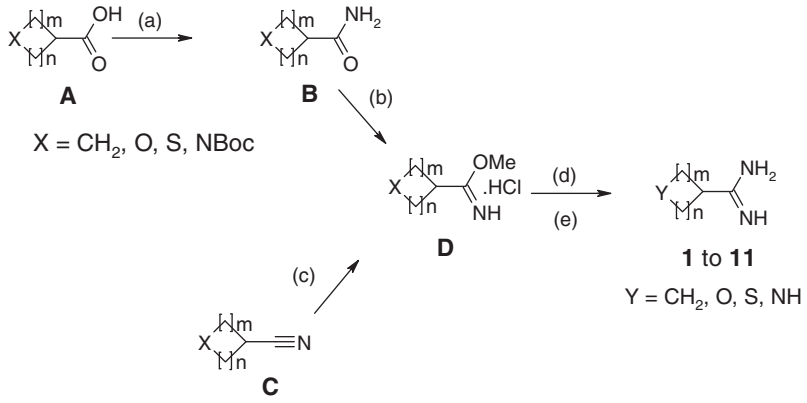
ported to interact in this manner[16] and some (aromatic) heterocyclic amidines have been made to probe for such an interaction, but without apparent success.[17]

We report here on the synthesis and evaluation of heteroalicyclic carboxamidines (Fig. 1B) targeted from inspection of the NOS crystal structures[18] and data on pyrazolo[19] and carbocyclic[20] carboxamidines. Increased potency was sought by probing for additional interactions with the protein (type I interaction) or to reach the co-ordination sphere of the haem iron to enable type II interactions.

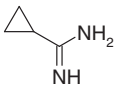
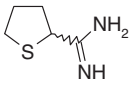
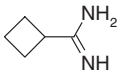
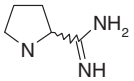
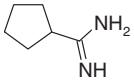
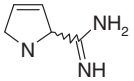
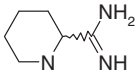
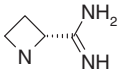
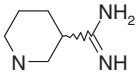
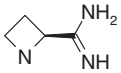
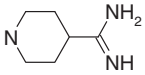
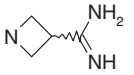
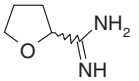
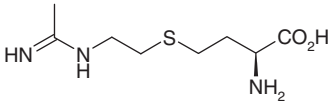
The synthetic strategy was to access carboxamidines (**2**–**15**) via the requisite imidate (**D**) derived from available acid (**A**), amide (**B**) or nitrile (**C**) precursors (Scheme 1). Specifically, acids (or N-Boc protected amino acids) **A** were converted to amides **B** by sequential treatment with carbonyl diimidazole then ammonia. Amides were converted to the requisite imidates **D** by treatment with trimethyloxonium tetrafluoroborate in dichloromethane. Alternatively, the imidates **D** were formed directly by reaction of nitriles with a saturated solution of hydrogen chloride in methanol. The reaction of imidates **D** with ammonium chloride in methanol gave the required amidines as hydrogen chloride salts in good yield after purification by reverse phase (C-18) solid phase extraction. As necessary, Boc protection was removed following amidination in 95:5 trifluoroacetic acid/water, affording the target compounds as trifluoroacetate salts after similar C-18 purification.

In vitro screening[21] against recombinant human iNOS[22] indicated that some significant increases in potency were achieved through our modifications (Table 1). Analysis of the SAR shows the importance of ring size, presence/position of a heteroatom and stereochemistry. Specifically, varying the ring size of carbocyclic amidines showed that cyclobutyl carboxamidine gave the best potency, closely followed by the smaller cyclopropyl analogue. Expansion to cyclopentyl carboxamidine resulted in a substantial loss of potency. The introduction of a heteroatom in the 2-position could dramatically influence activity; in the case of oxygen **7** or sulphur **8** there is little change compared with the carbocyclic **3**,



Scheme 1. (a) CDI, NH_3, DMF (b) Me_3OBF_4, DCM (c) $HCl_{(g)}$, MeOH (d) NH_4Cl, MeOH; (e) TFA/H_2O (95:5) if required. Compound **1** was purchased; compounds **3**, **7** and **10**–**13** were made from available acids (**A**), **4**, **5**, **6**, **9**, **14**, **15** from amides (**B**) and **2**, **8** from nitriles (**C**). All compounds gave satisfactory analytical data (LSMS/^{1}H NMR) supportive of the proposed structures and at least >95% purity.

Table 1
Human iNOS IC_{50} values[21] for a variety of ring systems, with **GW274150**[10] as a comparison.

Entry	Compound	hiNOS IC_{50} (μM)	Entry	Compound	hiNOS IC_{50} (μM)
1		0.79	8		5.3
2		0.38	9		0.7
3		4.4	10		0.18
4		>100	(***R***)-**11**		0.03
5		51	(***S***)-**12**		0.2
6		58	13		14
7		8.9	GW 274150		1.4

but nitrogen had a profound effect on increasing activity in four- and five-membered rings compared with their carbocyclic analogues. The physical basis of why nitrogen acted as a better ligand for iron than oxygen or sulphur was not established, given structural similarities and availability of lone pairs.

Of most significance was the impact of stereochemistry on activity—most notably in the big difference exhibited by the enantiomeric pyrrolidine derivatives, (***R***)-**14** and (***S***)-**15** (Table 2). The *R*-isomer was shown to be 100-fold more potent than the *S*, with an IC_{50} value of 0.12 μM. These compounds were also highly selective—the more potent enantiomer being >100-fold selective over both eNOS and nNOS. (Table 2)

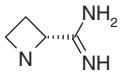
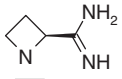
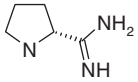
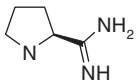
Docking studies of these compounds within a model of the iNOS active site were conducted using GOLD (Genetic Optimisation for Ligand Docking).[23] The model was built based on X-ray co-ordinates of the Δ114 monomer and published stereoscopic pictures of the Δ65 dimer available at the time.[24] The preferred docked poses suggested a clear rationale for the difference between (***R***)-**14** and (***S***)-**15**. With the expected hydrogen bonds between Glu_{377}/Trp_{372} and the mono-protonated carboxamidine in place, the neutral ring nitrogen of (***R***)-**14** could be located in close proximity to the haem iron (Fig. 2). This atom could therefore be acting as the sixth ligand to the metal. Physical measurements on (***R***)-**14** indicated that the amidine pK_a was 9.2 and that for the ring nitrogen 4.8; consistent with the ring nitrogen being essentially unionised at pH 7.4, thus presenting a lone pair to co-ordinate with iron. The docking of (***S***)-**15** in this manner indicated the requirement of a conformational shift in the protein structure to accommodate the ring.

Further evidence to support this hypothesis was provided by the data on the azetidines, (***R***)-**11** and (***S***)-**12**. These compounds also showed variation in activity between the enantiomers, but there was only 10-fold difference in potency. This is consistent with binding in a similar fashion to that proposed for the pyrrolidines, but as the azetidines are smaller, any conformational shift required for the *S*-isomer is less sterically demanding.

The most compelling evidence to support the postulated binding mode was secured through an examination of UV spectral changes obtained in competitive binding experiments using the Δ65-iNOS construct.[25] Addition of both (***R***)-**14** and (***S***)-**15** to the enzyme caused clear spectral shifts consistent with type II (Iron) binding.[26] The ***R***-enantiomer clearly bound with much greater affinity (binding Kd for (***R***)-**14** was 0.63 ± 0.16 μM) than the ***S***-(Kd >40 μM for (***S***)-**15**), consistent with the modelling predictions. However, the selectivity of the compound cannot be rationalised by possible steric interactions around the binding site in the constitutive isoforms, suggesting an alternative rationale; indeed the selectivity of amino acid amidine derivatives[9,10] is clearly not based on steric factors given the high local sequence homology and small size of the inhibitors.[27] It might be that subtle differences in redox chemistry across the isoforms, which give rise to selectivity in substrate based analogues,[28] contribute to this.

In conclusion, (2*R*)-2-pyrrolidinecarboxamidine, (***R***)-**14**, is a highly potent and highly selective inhibitor of iNOS. It is more potent than cyclopropyl carboxamidine and has a level of selectivity unprecedented in other simple amidines and guanidines. The increases in potency were rationalised by an interaction with the haem functionality, presenting an important improvement in inhibitor design. It is interesting to note that (***R***)-**14**, with a *c* log *P* of −0.93 and eight heavy atoms (HA) has a ligand efficiency [−RT ln(IC_{50})/HA] of 1.22 and the Lipophilicity Ligand Efficiency (pIC_{50} − *c* log *P*) is 7.9.

Table 2
IC_{50} values[21] and selectivity data for human NOS isoforms measured for single enantiomers of **9** and **11**, with **GW274150**[10] as a comparison

Entry	R	IC_{50} (μM)			Selectivity[a]	
		iNOS	eNOS	nNOS	i/e	i/n
(***R***)-**11**		0.03	0.25	0.26	8	9
(***S***)-**12**		0.2	2.4	1.7	12	9
(***R***)-**14**		0.12	15	14	125	117
(***S***)-**15**		9.4	>100	>100	>10	>10
GW 274150	—	1.4	466	145	333	104

[a] Selectivity expressed as the ratio of the IC_{50} of eNOS or nNOS to iNOS.

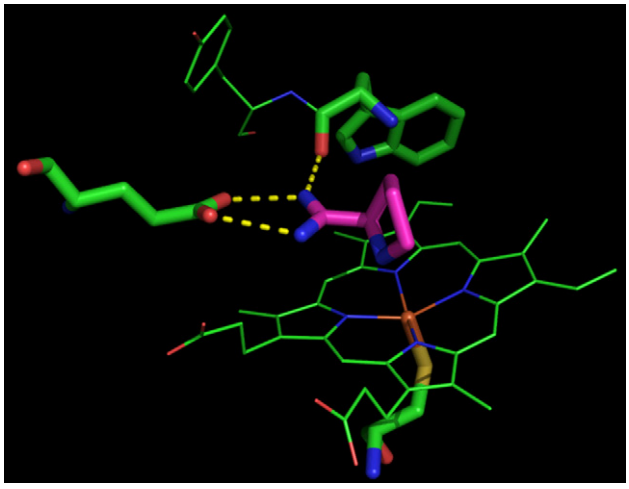


Figure 2. Docked pose of (***R***)-**14** in the active site model of iNOS (rendered using Pymol from iNOS structural data).

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26. The measured Kd for (***R***)-**14** was 0.63 ± 0.16 μM, with saturation of spectral changes at 10.5 μM. 40 μM of (***S***)-**15** showed only a minor shift in the spectrum from type I to type II and higher concentrations caused precipitation of the protein.
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