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The discovery of novel, potent and highly selective inhibitors of inducible nitric oxide synthase (iNOS)

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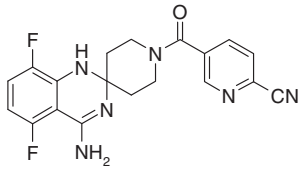
ABSTRACT

By careful analysis of experimental X-ray ligand crystallographic protein data across several inhibitor series we have discovered a novel, potent and selective series of iNOS inhibitors exemplified by compound **8**.

Nitric oxide (NO), produced from L-arginine by the nitric oxide synthases, plays a crucial role in cellular signalling. Over-production of NO is associated with a number of debilitating diseases; numerous reports implicate excess NO––from the inducible nitric oxide synthase isoform (iNOS)––in inflammatory conditions such as rheumatoid arthritis and asthma.[1–4] Potent, selective and safe small molecule inhibitors of iNOS would be valuable tools in further understanding the potential of inhibiting this enzyme to deliver therapeutic agents. We have previously reported on a variety of competitive iNOS inhibitors containing a *cis*-amidine motif, which mimics the guanidinium of L-arginine.[5] These investigations resulted in the discovery of the potent, and iNOS selective spirocyclic 1,2-dihydro-4-quinazolinamines exemplified by **1** (AR-C102222).



1 (AR-C102222)

Whilst the in vivo pharmacokinetic properties of **1** were suitable for establishing efficacy in a variety of disease models[5–7] **1** is reactive toward glutathione (γ-L-glutamyl-L-cysteinylglycine); this raises the possibility of reactive metabolite formation and idiosyncratic adverse drug reactions in man. We concluded that a new direction was required as we had explored the full potential of competitive inhibitors containing the amidine motif. We set out to investigate non-amidine inhibitors of iNOS in the hope that this might provide a better starting point for a program aimed at producing inhibitors with drug-like properties.

The search for non-amidine iNOS inhibitors presented two major challenges. The first was to maintain potency despite dispensing with the charge reinforced bi-dentate hydrogen bond between the amidine and the conserved glutamic acid (Glu377[8]). The second challenge was to understand and maintain the selectivity against the endothelial NOS (eNOS) in order to avoid unwanted blood pressure effects.[9] The origins of the selectivity we had achieved in **1** were, at the time, a subject of heated debate.[10] AR-C102222 **1** is greater than 2500-fold selective for iNOS versus eNOS even though the three isoforms share almost complete amino acid conservation, and structural similarity, around the arginine binding site.

Our hypothesis was that potency could be retained by keeping an aromatic ring over the haem group, and selectivity could be engineered by occupying the same binding site region accessed

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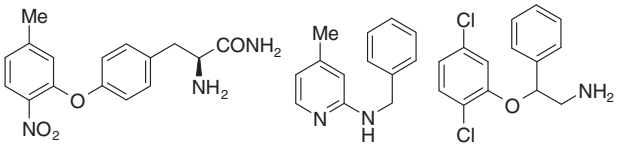
by **1**. To expedite this we screened other similar non-amidines whereupon compound **2**[11] was revealed as a weak inhibitor (IC_{50} 1.2 μM), but with little selectivity. A crystal structure showed that the amino-carboxamide of **2** resides in the main channel of the enzyme cavity. The nitro-group displaces the conserved Glu377 which swings to point away from the haem to expose its side-chain methylenes, thus forming a ligand induced binding pocket. The nitro-group appears to hydrogen bond to the backbone–NH of Met374 amide, which had previously bonded to the Glu377 carboxylate. A number of non-amidine small molecules have since been reported to bind to the NOS enzymes using this new pocket, for example substituted nitro-indazoles.[12,13] It is proposed that strong binding is achieved by stacking of the ligand over the haem and formation of a number of hydrogen bonds to the protein backbone. This binding is confirmed by X-ray crystal structures.



2 **3** **4**

The clue to establishing the required selectivity originated from the crystal structure of compound **3**. As expected the aminopyridine, an amidine mimic, binds to Glu377 in a similar way to L-arginine, but it was noticed that the *N*-benzyl substituent, by shifting the isoform conserved residue Gln263, forms a new pocket and gives this simple compound (**3**, IC_{50} 0.79 μM) a 12-fold selectivity against eNOS. Examination of a large number iNOS ligands co-crystallised with the oxygenase domain of mouse iNOS, and human or bovine eNOS, revealed that an isoform specific pocket was created by bulky inhibitors of human iNOS. Although Gln263 is invariant in the three NOS isoforms the key to specificity is the ability of the Gln263 residue in iNOS to shift and expand the binding pocket. Due to differences in the second shell of amino acids around the binding site this can only occur in the iNOS isoform.[10]

The combination of the fragments, retention of the basic amine (to bind the haem carboxylic acids) and replacement of the nitro-group resulted in **4**, which is gratifyingly both potent (IC_{50} 1.9 μM) and selective (50-fold against eNOS and nNOS, see Table 2). Compound **4** became the starting point for an optimisation program which culminated in the discovery of exciting novel and selective iNOS inhibitors.

The compounds were synthesised in essentially two steps (Table 1). The first was a coupling reaction (Scheme 1); either a Mitsunobu coupling (method A), or an aromatic displacement of a halogen (method B), both with a chiral alcohol. Depending on the chiral alcohol further steps (i–iv) were required to complete the synthesis.

Extension of the amino-methyl chain of compound **4** by one carbon atom, to provide propylamine **5**, results in a remarkable 90-fold improvement in potency; further chain homologation provided no additional benefit. Gratifyingly **5** is greater than 4500-fold selective over eNOS and 210-fold selective against nNOS (Table 2). However, this compound shows a very high drop in cellular potency of around 400-fold, which is much higher than the 50-fold expected due to competition with the intracellular concentration of the NOS enzyme's substrate L-arginine. As the molecule's high lipophilicity (calculated log $P = 4.1$) was thought to be responsible one of the two chlorine atoms in **5** was successfully replaced with a cyanide group providing improved cellular potency (**6a**, IC_{50} 0.7 μM). The selectivity of **6a** against eNOS was greater than 10,000-fold! The cyanide group also appears to make a hydrogen bond interaction with the backbone –NH of Met374 amide.

A crystal structure of **6b**, the des-*N*-methyl analogue of **6a**, shown in Figure 1[15] demonstrates that the compound binds with the aryl group over the haem, with the cyanide group in the shifted Glu377 pocket, and the benzyl group in the Gln263 shifted 'glutamine specificity pocket'. However, the position of the glutamine residue indicates that this pocket is similar to, but not exactly identical to that previously reported.[10]

Examining the chiral centre we found that the (R)-enantiomers were consistently 10 to 100-fold more potent than the (S) enantiomers––this was confirmed periodically with key compounds and crystal structures. Changing the linking atom from oxygen **6b** to sulphur **6c** improved iNOS potency, but was accompanied by an unacceptable reduction in nNOS selectivity; this observation is general to the series. More sterically demanding non-polar groups, for example trifluoromethyl **6d** or methoxy **6e,** are tolerated, but with some loss of potency. Such large groups probably describe the limit in size for substituents at the C-3 position (R^1). Moving the C-3 substituent to the C-4 (R^2) position, as in **6f**, removed much of the iNOS potency. Replacing the phenyl ring––which projects toward the Gln263 pocket––with a small alkyl chain **6g** lowers potency and removes most of the eNOS selectivity. This supports the original hypothesis that selectivity is only achieved by moving the glutamine residue in iNOS.

Compound **6a** is stable in human microsomal and hepatocyte preparations, but is not stable in rat hepatocytes; accordingly it is not bioavailable when dosed orally to a rat. The principal routes of metabolism were identified as N-de-methylation and oxidation at C-4. These metabolic routes were blocked by substituting the C-4 hydrogen atom with fluorine, and removing the N–Me group to

Table 1
Structures and synthesis methods

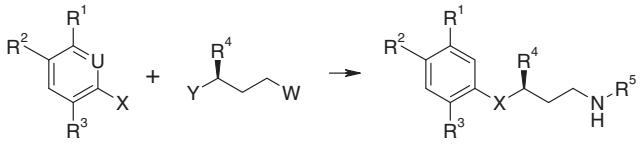
Compd	Structure							Synthesis				
	U	R^1	R^2	R^3	R^4	$R^{5,a}$	X	Y	W	Coupling method	Final step	Salt[b]
5	C	Cl	H	Cl	Ph	Me	OH	O	–NMeBoc	A	i	$C_4H_4O_4$
6a	C	Cl	H	–CN	Ph	Me	OH	O	–Cl	A	ii	HCl
6b	C	Cl	H	–CN	Ph	H	F	O	$–N_3$	B	iii	$C_2H_2O_4$
6c	C	Cl	H	–CN	Ph	H	F	S	$–N_3$	B	iii	$C_4H_4O_4$
6d	C	$–CF_3$	H	–CN	Ph	Me	F	O	–NMeBoc	B	i	HCl
6e	C	MeO–	H	–CN	Ph	Me	OH	O	–NMeBoc	A	i	$C_2H_2O_4$
6f	C	H	Cl	–CN	Ph	H	OH	O	Cl	A	iv	$C_4H_4O_4$
6g	C	Cl	H	–CN	Et	Me	F	O	–NMeBoc	B	i	HCl
6h	C	Cl	F	–CN	Ph	H	F	O	$–N_3$	B	iii	$C_2H_2O_4$
7	N	Me	H	–CN	Ph	H	Cl	S	$–N_3$	B	iii	$C_4H_4O_4$
8	N	$–CF_3$	H	–CN	5-Isoxazoyl	H	Cl	O	–NHBoc	B	i	$C_2H_2O_4$

[a] Stereochemistry is R as drawn, for all compounds except **6g** which is racemic.
[b] Salts were prepared by (neutralising if required) adding the relevant acid (1 equiv)––fumaric ($C_4H_4O_4$), oxalic ($C_2H_2O_4$) acid or HCl in a suitable solvent at room temperature.

Table 2
Biological activities and selectivity profiles

	log *D*	iNOS (μM)[a]	Sel. eNOS[a]	Sel. nNOS[a]	Cell (μM)[a]	SET (μM)	NA (μM)	CY2D6 (μM)	hERG (μM)
1	0.79	0.041	>3000	22	1	NA	NA	NA	0.1
4	NA	1.9	>53	52	NA	NA	NA	NA	NA
5	NA	0.022	>4500	210	8.7	0.0	0.0	NA	NA
6a	1.7	0.0090	>10,000	140	0.7	4.5	0.5	1.0	10
6b	0.8	0.0040	>10,000	270	0.9	2.1	15.0	0.4	NA
6c	1.3	0.0040	>10,000	33	0.2	6.7	17.0	4.1	NA
6d	1.3	0.071	1400	5	2.7	10.0	28.0	1.6	NA
6e	0.9	0.110	NA	65	9.5	5.7	12.0	0.3	NA
6f	0.9	0.960	100	104	NA	NA	NA	NA	NA
6g	0.4	0.051	170	18	3.6	44.0	7.1	0.4	NA
6h	0.8	0.0060	>10,000	35	0.7	4.4	16.0	0.34	>10
7	0.7	0.0040	>10,000	28	0.6	2.6	7.4	5	NA
8	−0.5	0.0054	>10,000	1.3	0.72	70	>100	>14	>10

[a]For nitric oxide enzyme and cellular screening methods.[5,14]



Scheme 1. Synthesis of compounds **5**–**8**. Coupling method A: Ph_3P, DEAD, THF, 0–25 °C 12 h. Coupling method B: Either NaH (1 equiv), in DMF (or THF), 1 h, 40 °C; or, K_2CO_3 in DMF (or MeOH), 40 °C. Final steps: (i) 4 N HCl, in 1,4-dioxane (or THF), 25 °C; (ii) NaI, acetone, reflux, 18 h then 40% aq $MeNH_2$, 25 °C, 5 h; (iii) Ph_3P, 10% aq THF (v/v), reflux, 1 h; (iv) NaN_3, DMSO, 60 °C 16 h then Ph_3P, 10% aq THF (v/v), reflux, 1 h.

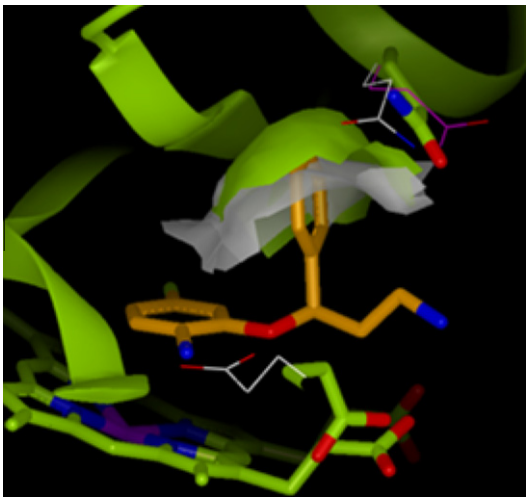


Figure 1. X-ray structure of **6b**[15] into the oxygenase domain of mouse iNOS, resolution 2.65 Å. The Glu371 and Gln257 (mouse numbering) positions when L-arginine is bound are shown in white. This emphasises the movements of these residues on binding of our non-amidine inhibitors. Surface representations in white and green illustrate the effect the position of Gln257 has on the size of the binding pocket. The Gln257 position found in the work reported previously for amidine like inhibitors is shown in purple.[10]

give the primary amine **6h**. As expected a marked improvement in the half-life and bioavailability (Table 3) was observed. Whilst **6h** maintained cellular potency, selectivity over nNOS was somewhat reduced; this observation is seen in a number of analogues containing fluorine at C-4. To address concerns about reactive metabolites rats were dosed with radio-labelled **6h**; no metabolites consistent with displacement of either the fluorine or chlorine by glutathione were observed. Further profiling revealed that **6h** is a

Table 3
Pharmacokinetic profile of compounds **1**, **6a**, **6h** and **8**

Compd	Rat	Human		Rat dosed intravenously			Rat dosed orally
	Heps[a]	Mics[a]	Heps[a]	Cl[b]	Vd_{ss}[c]	$T_{1/2}$ (h)	Bioavailability (%)
1	5	NA	<3	57	4.5	1.1	75
6a	54	4	1.4	94	6.6	1.3	NA
6h	8.9	6	<1	49	13	4.3	57
8	15	<3	1	56	3.4	1.0	49

[a] Mics: microsomes, units of μL/min/mg. Heps: hepatocytes, units of μL/min/10^6 cells.
[b] Clearance of drug from plasma, units of mL/min/kg.
[c] Volume of distribution at steady state, units of L/kg.

potent CYP2D6 inhibitor (Table 2, IC_{50} 0.34 μM); this proved a general property of the series. These new iNOS inhibitors bear a significant structural resemblance to the selective serotonin reuptake inhibitor Fluoxetine™, and whilst Fluoxetine™ is not an inhibitor of any of the three NOS isoforms these compounds do bind to the noradrenaline (NA) and serotonin transporters (SET). Compounds were also tested for hERG activity (Table 2), but were shown to be very weak inhibitors.

Removal of these unwanted properties was achieved simply by lowering the compounds' lipophilicity. This was initially attempted by replacing the phenyl ring over the haem with a pyridine analogue **7** which retained potency and selectivity for iNOS, but did not abrogate the SET or NA binding. A meaningful reduction in lipophilicity was achieved by replacing the phenyl ring projecting toward the Gln263 pocket with a heterocyclic ring. The combination of a 3-trifluoro-2-pyridyl group and an isoxazole gave **8** which is a potent and selective iNOS inhibitor, and gratifyingly removed the NA, SET and CYP2D6 activities. The only shortcoming of **8** was the lack of nNOS selectivity; this is likely due to the smaller size of the isoxazole (compared with the phenyl substituent) binding in the 'glutamine selectivity pocket'.

A feature of this series of novel iNOS inhibitors is their stability in in vitro metabolising systems (human and rat), and their suitability for in vivo testing in rat models on inflammatory disease (Table 3). The series had rat half lives of greater than 1 h with moderate to good bioavailability after oral dosing. A significant fraction of drug is free in plasma, and all have acceptable solubility in buffered aqueous media under equilibrium conditions at 37 °C. Compound **6h** demonstrated significant efficacy in a collagen induced arthritis disease model, in female Dark Agouti rats, when dosed twice daily at 75 μmol/kg. Pronounced analgesic activity was observed, and histological analysis showed significant reduction in inflammation and erosion scores.[16]

In conclusion we have demonstrated that careful analysis of experimental crystallographic ligand–protein data across several inhibitor series allowed a novel, potent and selective series of iNOS inhibitors, exemplified by **6h** and **8**, to be designed. The series has excellent properties for further investigation of the role of inducible nitric oxide synthase in inflammatory disease models. Further publications will discuss the journey to provide compounds with a profile more suitable for human dosing.

Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmcl.2011.02.061.

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14. *For the preparation of compounds, excepting* **6f** *and* **4**, *including structural data and screening methods*: Compounds **5**, **6a**–**b**, **6d** and **6h**: Cheshire, D.; Connolly, S.; Cox, D.; Hamley, P.; Mete, A.; Pimm, A. WO2001062704, **2001**; **6c**, Cheshire, D.; Connolly, S.; Cox, D.; Millichip, I. WO 2001062721, **2001**: **6g**, Cheshire, D.; Connolly, S.; Cox, D.; Mete, A. WO2001062714, **2001**; **6e**, Cheshire, D.; Connolly, S.; Cox, D.; Hamley, P.; Mete, A.; Pimm, A. WO2001062704, **2001**; **7** and **8**, Birkinshaw, T.; Cheshire, D.; Connolly, S.; Luker, T.; Mete, A. WO2003011830, **2003**. Compounds **6f** and **4** were made using the methods described in these references. A procedure for **6h** can be found in the Supplementary Data.
15. RCSB Protein Data Bank; entry code 2Y37.
16. Unpublished data.