Brief Articles

2-Aminopyridines as Highly Selective Inducible Nitric Oxide Synthase Inhibitors. Differential Binding Modes Dependent on Nitrogen Substitution

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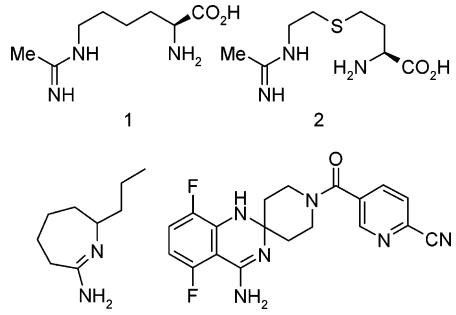
4-Methylaminopyridine (4-MAP) (**5**) is a potent but nonselective nitric oxide synthase (NOS) inhibitor. While simple N-methylation in this series results in poor activity, more elaborate N-substitution such as with 4-piperidine carbamate or amide results in potent and selective inducible NOS inhibition. Evidently, a flipping of the pyridine ring between these new inhibitors allows the piperidine to interact with different residues and confer excellent selectivity.

Introduction

In the past decade, nitric oxide has been revealed as a signaling and effector molecule that plays diverse biological roles, depending on which of the three subtypes of the enzyme nitric oxide synthase (NOS) is involved in its biosynthesis and at which location.[1] Type I NOS (neuronal, nNOS) plays a role in skeletal muscle relaxation and cerebral blood flow, type III NOS (endothelial, eNOS) relaxes smooth muscle in the vasculature through activation of cGMP, and type II (inducible, iNOS) is expressed and activated during inflammatory events. It has been proposed for some time that expression (or overexpression) of iNOS contributes to the progression of inflammatory diseases such as rheumatoid arthritis and osteoarthritis.[2] Selective inhibition of iNOS would therefore be a useful therapy for such diseases, leading to reduction of inflammation, protection of the joint toward erosion, and possibly alleviation of the associated pain. Indeed, results in animal models of arthritis with nonoptimal inhibitors support this hypothesis.[2] However, to date, only one class of compounds has been reported to have entered clinical testing in man.[3]

The class of iNOS inhibitors that have come closest toward having the requisite potency and isoenzyme selectivity are the arginine analogues such as L-iminoethyl lysine (L-NIL) **1**[3a] (which has entered clinical evaluation in man as its tetrazole−amide acid prodrug L-NILTA[3b]) and GW271450 **2**[3c] (Table 1). In the case of L-NIL, selectivity against the other isoenzymes, particularly nNOS, is poor, while in both amino acid inhibitors potency is quite weak. Because of their physicochemical properties, amino acid arginine-like inhibitors will rely on transport processes for their absorption and distribution. Progress has been made in the effort to discover more druglike inhibitors of iNOS by moving away from the amino acid template and has resulted in simple cyclic amidines such as **3**[4] and the recently described bicyclic and spirocyclic amidines such as **4**.[5] However, there is still a need for potent and selective inhibitors of iNOS that would be more druglike and suitable for progression to the market as drugs.

Table 1. Comparison of Activity of Various Published iNOS Inhibitors



compd	class of inhibitor	IC_{50} (μM) iNOS[a]	IC_{50} (μM) eNOS[b]	IC_{50} (μM) nNOS[c]
1	amino acid amidine	0.13	2.4	1.6
2	amino acid analogue	0.070	34	18
3	cyclic amidine	0.026	4.8	0.24
4	spirocyclic amidine	0.037	>100[d]	0.74

[a] Inhibition of human-induced NOS. [b] Inhibition of human endothelial NOS. [c] Inhibition of human neuronal NOS. [d] Less than 20% inhibition at 100 μM.

Results

The substrate for all three NOS enzymes is L-arginine. The first crystal structure of the iNOS oxidase

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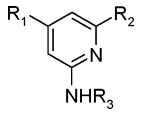
Table 2. Inhibition of iNOS by Simple Aminopyridine Derivatives



compd	R_1	R_2	R_3	IC_{50} (μM) iNOS[a]	IC_{50} (μM) eNOS[b]	IC_{50} (μM) nNOS[c]
5	Me	H	H	0.12	0.30	0.11
6	Me	Me	H	0.045	0.17	0.098
7	Me	Pr	H	0.01	0.05	0.01
8	Me	Me	Me	86	30	>100[d]
9	Et	H	H	1.1	4.3	2.2
10	MeO	H	H	1.0	1	1.0

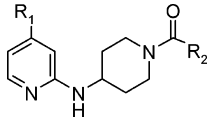
[a] Inhibition of human-induced NOS. [b] Inhibition of human endothelial NOS. [c] Inhibition of human neuronal NOS. [d] Less than 20% inhibition at 100 μM.

domain[6] revealed a critical role for the residue Glu[371] in forming charge-reinforced hydrogen bonds with hydrogens attached to two of the nitrogen atoms of the guanidinium moiety, allowing oxidation of the third guanidine N-atom of L-Arg to occur over the heme iron. It is therefore not surprising that most competitive NOS inhibitors to date have contained a minimum pharmacophore of a basic *cis*-amidine (an isostere of the substrate guanidine moiety) required for a bidentate interaction with the carboxyl group of Glu[371]. With this pharmacophore in mind, a large number of 2-aminopyridines were screened that contained an appropriate amidine substructure (Table 2). The most potent inhibitor discovered from this exercise was the simple 4-methylaminopyridine **5**, although this compound has little selectivity. Subsequently it was found that simple alkyl groups in the 6-position increase potency (as in **6** and **7**) but still confer no selectivity. N-methylation appears to destroy activity completely (compare **6** and **8**), while increasing the size of the 4-substituent to either Et or MeO (**9** or **10**) reduces potency significantly. A 4-methyl group appeared to be the optimal substitution on the pyridyl ring.[7]

Despite the drastic effect of N-methylation of the exocyclic NH_2-group of compound **7**, a simple parallel synthesis program was undertaken to explore a wider range of substituents on the amino group. Compounds were readily synthesized by Buchwald palladium-catalyzed aromatic amination.[8]

Surprisingly, incorporation of a 4-piperidinyl carbamate moiety, as in **11** (Table 3), gives an inhibitor that has potency similar to that of 4-MAP (**5**) but has vastly improved selectivity against the other subtypes. When the pyridine 4-methyl group of **11** was varied, the corresponding 4-unsubstituted analogue **12** had much reduced potency, further suggesting that a 4-methyl group might be optimal. However, in contrast to the structure−activity relationship established in the 6-substituted aminopyridine series, in this new N-substituted aminopyridine series replacing the 4-methyl with 4-methoxy (**13**) surprisingly gave a 4-fold improvement in potency against iNOS. Furthermore, since the potency against nNOS and eNOS was not improved, selectivity of **13** against both isoenzymes is very high (>450-fold vs eNOS and >350-fold vs nNOS). Increasing the size of the 4-substituent even further from methoxy to ethoxy, as in **14**, produced a 50-fold drop in potency against iNOS, indicating a new size-limited binding requirement for which a methoxy group appears to be optimal.

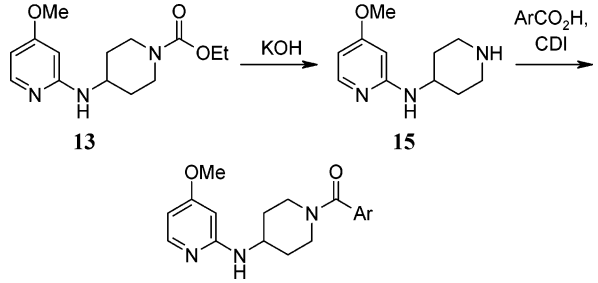
Table 3. Selective Inhibition of iNOS by *N*-4-Piperidinyl-2-aminopyridine Derivatives



compd	R_1	R_2	IC_{50} (μM) iNOS[a]	IC_{50} (μM) eNOS[b]	IC_{50} (μM) nNOS[c]
11	Me	OEt	0.35	50	21
12	H	OEt	13	>100[d]	>100[d]
13	MeO	OEt	0.089	41	32
14	EtO	OEt	3.8	>100[d]	96
16	MeO	Ph	0.53	100	28
17	MeO	4-ClPh	0.053	36	7.8
18, AR-C133057XX	MeO	4-CNPh	0.071	>100[d]	6.6

[a] Inhibition of human-induced NOS. [b] Inhibition of human endothelial NOS. [c] Inhibition of human neuronal NOS. [d] Less than 20% inhibition at 100 μM.

Scheme 1. Parallel Synthesis of *N*-4-Piperidinyl-2-aminopyridine Benzamide Inhibitors of iNOS



Compound **13** was further optimized by replacing the carbamate with an aromatic amide. Since the synthesis of these compounds was amenable to rapid parallel chemistry, a large number of variations were made in parallel by the route described in Scheme 1. The ethyl carbamate **13** was hydrolyzed to give the free piperidine **15**, and this amine was acylated in a parallel fashion with a variety of arylcarboxylic acids. While the simple benzamide **16** was less potent than carbamate **13**, 4-substitution on the benzamide was found to lead to the most potent compounds. For example, 4-chlorobenzamide **17** and 4-cyanobenzamide **18** were as active as the parent carbamate while still retaining good selectivity against the other NOS enzymes. In addition, these arylamides had much better in vivo metabolic stability (rat) than the carbamate **13**. Thus, the combination of a 4-MeO group on the pyridine ring and a 4-cyano group on the benzamide resulted in **18** (AR-C133057XX), a compound with good potency and that has >1400-fold selectivity vs eNOS, and ~100-fold selectivity against nNOS.

Discussion and Conclusions

To cast light on the differences observed in SAR between the N-unsubstituted and N-substituted series, X-ray crystal structures of 4-MAP (**5**) and **18** complexed with the mouse iNOS oxidase domain dimer were compared (see Figure 1). As expected, the aminopy-

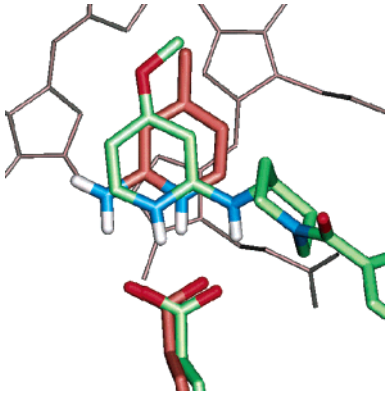


Figure 1. Overlay of the X-ray crystal structures of the heme-binding regions of 4-MAP and AR-C133057XX showing the aminopyridine ring-flip.

ridines in both compounds bind in a two-point charge-reinforced interaction with the carboxylate group of Glu^{371}, with the pyridine aromatic rings lying over the heme unit. Intriguingly**,** *these pyridine rings are flipped across the pyridine plane* so that the two nitrogens of each compound bind to the opposite oxygen of the glutamic acid residue. The methyl group of the 4-methyl substituent and the methyl group of the 4-methoxy group on the pyridine rings occupy the same area of space in the two inhibitors, accounting for the different SAR observed in the two series and suggesting this lipophilic interaction within the heme pocket is important for potency. In the 6-substituted aminopyridine iNOS inhibitor **5**, the exocyclic NH_2 group binds deeper in the heme pocket than the (protonated) pyridine N-atom, and any further substitution is necessarily limited to the sp^2 6-position, making the search for selectivity limited and synthetically difficult. In contrast, in the new N-substituted aminopyridine iNOS inhibitor **18**, the (protonated) pyridine N-atom binds deeper in the heme pocket while the exocyclic NH_2 group is pointing out of the heme pocket and is accessible for easy and varied linking to ancillary binding pockets. Presumably it is this extra binding that gives rise to the good selectivity observed.

In summary, new potent and highly selective N-substituted aminopyridine iNOS inhibitors have been identified that have druglike properties. Crystallographic structures suggest a fascinating difference in binding mode between these new compounds and simpler N-unsubstituted analogues. This unexpected flipped amidine binding mode allows easy incorporation of additional binding groups to the aminopyridine template and has led to excellent selectivity against the other NOS enzymes. It is interesting to note that the early indications suggested that substitution on the exocyclic amino group would not lead to active compounds. However, with the insight provided by X-ray crystallography, this observation can easily be rationalized.

Experimental Section

General Procedure for the Preparation of N-Substituted Aminopyridines 11−14. BINAP (0.5 mmol), palladium(II) acetate (0.5 mmol), and 2-bromo- or 2-chloropyridine (10 mmol) were dissolved in dry toluene (50 mL). Ethyl 4-amino-1-piperidinecarboxylate (12 mmol) was added, followed by potassium *tert*-butoxide (14 mmol), and the mixture was heated at 70 °C for 4 h. The mixture was cooled to room temperature, quenched with water, and extracted twice with ethyl acetate. The organic extract was dried, the solvent was evaporated, and the residue was purified by flash chromatography (ethyl acetate/hexane). The resulting colorless gum was dissolved in methanol (5 mL), treated with HCl (4 M in dioxane, 1 mL) or maleic acid (1 equiv), and stirred for 10 min. The solvent was evaporated and the residue was triturated with ether to give the product.

4-[(4-Methoxy-2-pyridinyl)amino]piperidine (15). A solution of the carbamate **13** (12.3 g, 44 mmol) in ethylene glycol (130 mL) and 3 M aqueous potassium hydroxide (65 mL) was heated for 16 h. The mixture was cooled, basified with additional 3 M aqueous potassium hydroxide, and washed with ethyl acetate. The organic extract was evaporated, dried, and concentrated to a thick oil. Trituration with ethyl acetate/diethyl ether gave the product as a cream solid (6.0 g): ^1H NMR (400 MHz, $CDCl_3$) δ 7.9 (1H, d), 6.19 (1H, dd), 5.84 (1H, d), 4.6 (1H, d), 3.79 (3H, s), 3.67 (1H, m), 3.15 (2H, dt), 2.77 (2H, m), 2.07 (2H, m) 1.42 (2H, m); MS (+CI) *m/z* 208 ($M^+ + 1$, 100%).

General Procedure for the Acylation of Piperidine 15 with Benzoic Acids. To the benzoic acid (0.56 mmol) in DMF (4 mL) was added carbonyl diimidazole (0.67 mmol) followed after 20 min by (4-methoxypyridin-2-yl)piperidin-4-ylamine (0.56 mmol). After 30 min, the mixture was diluted with water, extracted with ethyl acetate, dried, and evaporated. The compound was purified by normal-phase HPLC, eluting with dichloromethane/ethanol (95:5), and the residue was triturated with ether to give the product.

Supporting Information Available: General procedures, in vitro assay details, spectroscopic information, and elemental analyses for all test compounds. This material is available free of charge via the Internet at http://pubs.acs.org.

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