Expedited Articles

2-Iminopiperidine and Other 2-Iminoazaheterocycles as Potent Inhibitors of Human Nitric Oxide Synthase Isoforms†

William M. Moore,*,‡ R. Keith Webber,§ Kam F. Fok,§ Gina M. Jerome,‡ Jane R. Connor,‡ Pamela T. Manning,‡ Pamela S. Wyatt,‡ Thomas P. Misko,‡ Foe S. Tjoeng,§ and Mark G. Currie‡

Departments of Molecular Pharmacology/Inflammatory Diseases Research and Medicinal Chemistry, G. D. Searle Research and Development, Monsanto Company, Mail Zone T3G, 800 North Lindbergh Boulevard, St. Louis, Missouri 63167

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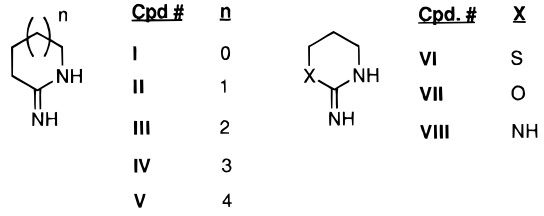
A series of 2-iminoazaheterocycles have been prepared and shown to be potent inhibitors of human nitric oxide synthase (NOS) isoforms. This series includes cyclic amidines ranging from five- to nine-membered rings, of which 2-iminopiperidine and 2-iminohomopiperidine were the most potent inhibitors, with IC_{50} values of 1.0 and 2.0 μM, respectively, for human inducible nitric oxide synthase. This series of cyclic inhibitors was further expanded to include analogs with heteroatoms in the 3-position of the six-membered ring. This modification was tolerated for sulfur and oxygen, but nitrogen reduced the inhibitory potency. The oral administration of 2-iminopiperidine in lipopolysaccharide (LPS)-treated rats inhibited the LPS-induced increase in plasma nitrite/nitrate levels in a dose-dependent manner, demonstrating its ability to inhibit inducible NOS activity *in vivo*. These cyclic amidines represent a new class of potent NOS inhibitors and the foundation for potential therapeutic agents.

Introduction

Nitric oxide synthase (NOS) catalyzes the oxidation of L-arginine to produce L-citrulline and the biologically active free radical nitric oxide. Several recent reviews describe the progress that has been made in the study of NOS and the biological activity of nitric oxide.[1−5] In general there are two major classes of NOS: constitutive and inducible. The constitutive enzymes require Ca^{2+}/calmodulin for activity and are further divided into neuronal and endothelial isoforms. The endothelial isoform is found predominantly in the vascular endothelium and generates low concentrations of nitric oxide which lowers blood pressure and inhibits platelet aggregation. Nitric oxide generated by the neuronal enzyme appears to function as a neurotransmitter regulating neuronal transmission. The inducible isoform is found in activated macrophages as well as many other cell types and produces nitric oxide which plays a role in host defense. It is this form of the enzyme that is implicated in the excessive production of nitric oxide that destroys functional tissue during acute and chronic inflammation.

Identification of potent and selective inhibitors of the inducible form of NOS has been the subject of intense interest because of their therapeutic potential for the treatment of diseases mediated by excessive production of nitric oxide.[6,7] Structural analogs of L-arginine such as N^G-monomethyl-L-arginine (L-NMA),[8,9] N^G-nitro-L-arginine (L-NNA),[10] L-N^5-(1-iminoethyl)ornithine (L-NIO),[11] and L-thiocitrulline[12] have been shown to be inhibitors of the various forms of NOS but lack selectivity for the inducible isoform. We have recently reported that L-N^6-(1-iminoethyl)lysine (L-NIL) is a potent and selective inhibitor of mouse inducible NOS and suppresses the increase in plasma nitrite/nitrate levels and joint inflammation associated with adjuvant arthritis.[13,14] Non-amino acid NOS inhibitors have been described and include aminoguanidine,[15] isothioureas,[16] and 2-amino-5,6-dihydro-6-methyl-4*H*-1,3-thiazine.[17] Here we report for the first time that 2-iminopiperidine (**II**) (Figure 1) and the homologous series of cyclic amidines are potent non-amino acid inhibitors of human NOS with modest selectivity for inducible NOS over the endothelial constitutive isoform.



Cpd #	n
I	0
II	1
III	2
IV	3
V	4

Cpd. #	X
VI	S
VII	O
VIII	NH

Figure 1. Structures of compounds **I**−**VIII**.

In evaluating compounds containing the amidino group as potential NOS inhibitors, we found that 2-iminopiperidine (**II**) was a potent inhibitor of human NOS isoforms, with IC_{50} values of 1.0 μM for hiNOS, 4.7 μM for hecNOS, and 1.1 μM for hncNOS (Table 1). In order to investigate the effect of ring size on potency and selectivity, we synthesized the corresponding five-, seven-, eight-, and nine-membered ring analogs and determined their potency and selectivity as NOS inhibitors. As shown in Table 1, 2-iminopiperidine (**II**) was the most potent of this series. The seven-membered ring compound, 2-iminohomopiperidine (**III**), had simi-

† Abbreviations: NOS, nitric oxide synthase; hiNOS, recombinant human inducible NOS; hecNOS, recombinant human endothelial constitutive NOS; hncNOS, recombinant human neuronal constitutive NOS; L-NIL, L-N^6-(1-iminoethyl)lysine; L-NIO, L-N^5-(1-iminoethyl)-ornithine; L-NNA, N^G-nitro-L-arginine; L-NMA, N^G-monomethyl-L-arginine; LPS, lipopolysaccharide.

* Author for correspondence. Tel: (314) 694-8951. Fax: (314) 694-8949.

‡ Molecular Pharmacology/Inflammatory Diseases Research.

§ Medicinal Chemistry.

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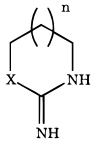
Table 1. Comparison of IC_{50} Values for Inhibition of Human NOS Isoforms



			IC_{50}[a] (μM)			selectivity[b]	
compd	*n*	X	hiNOS	hecNOS	hncNOS	hec/hi	hnc/hi
I	0	CH_2	26	93	20	3.6	0.8
II	1	CH_2	1.0	4.7	1.1	4.7	1.1
III	2	CH_2	2.0	16	3.5	8.0	1.8
IV	3	CH_2	10	59	10	5.9	1.0
V	4	CH_2	9.8	90	6.8	9.2	0.7
VI	1	S	2.9	7.1	3.2	2.4	1.1
VII	1	O	1.8	8.2	3.7	4.6	2.1
VIII	1	NH	25	87	17	3.5	0.7
L-NIL			4.6	138	61	30	13
L-NMA			14	5.9	10	0.4	0.7
L-NNA			7.6	0.5	0.5	0.066	0.066

[a] IC_{50} values were determined with hiNOS, hecNOS, and hncNOS by testing each compound at eight concentrations. NOS activity was measured in the presence of a final L-arginine concentration of 30 μM by monitoring the conversion of L-[2,3-^{3}H]arginine to L-[2,3-^{3}H]citrulline as described in the Experimental Section. [b] Selectivity is defined as the ratio of the IC_{50}(hecNOS) or IC_{50}(hncNOS) to IC_{50}(hiNOS).

lar potency with an IC_{50} of 2.0 μM for hiNOS. In comparison, potency for hiNOS was substantially less for the smaller five-membered ring 2-iminopyrrolidine (**I**) and slightly less with the larger eight (**IV**)- and nine (**V**)-membered ring analogs (Table 1). Both 2-iminopiperidine (**II**) and 2-iminohomopiperidine (**III**) were more potent for hiNOS than L-NMA, L-NNA, and L-NIL. Selectivity for hiNOS as compared to hecNOS is indicated by the ratio of IC_{50}(hecNOS) to IC_{50}(hiNOS). As seen in Table 1, the hecNOS/hiNOS selectivity ratios for compounds **I**−**V** range from 3.6 to 9.2 and generally tend to increase with increasing ring size. 2-Iminohomopiperidine (**III**) and 2-azacyclononanone imine (**V**) were the most selective with hecNOS to hiNOS IC_{50} ratios of 8 and 9.2, respectively. Both 2-iminopiperidine (**II**) and 2-iminohomopiperidine (**III**) were substantially more selective for hiNOS over hecNOS than L-NMA and L-NNA. Selectivity for hiNOS compared to hncNOS for compounds **I**−**V**, given in Table 1 as the ratio of IC_{50}(hncNOS) to IC_{50}(hiNOS), ranged from 0.7 to 1.8, indicating essentially little or no selectivity for hiNOS over the neuronal isoform.

In order to investigate the nature of inhibition of hiNOS by 2-iminopiperidine, a Lineweaver−Burk double-reciprocal plot analysis of the kinetics of hiNOS in the presence of different fixed concentrations of 2-iminopiperidine was performed. As shown in Figure 2, the double-reciprocal plots resulted in lines intersecting at the same point on the *y*-axis, indicating that 2-iminopiperidine is a competitive inhibitor of L-arginine binding to hiNOS. A K_i of 0.36 μM for 2-iminopiperidine was determined from a replot of the slope of each double-reciprocal plot versus the concentration of 2-iminopiperidine (data not shown).

We expanded this series of compounds with the preparation of analogs of 2-iminopiperidine with N, S, or O in the 3-position to afford 2-imino-5,6-dihydro-1,3-thiazine (**VI**), 2-imino-5,6-dihydro-1,3-oxazine (**VII**), and 2-iminotetrahydropyrimidine (**VIII**) (Figure 1). As shown

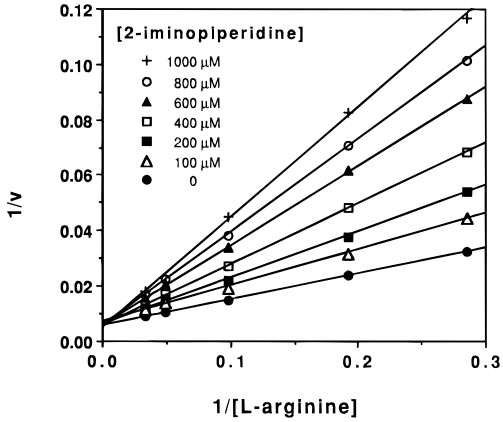


Figure 2. Plot of the reciprocal of hiNOS velocity (in units of min/pmol) versus the reciprocal of L-arginine concentration (in units of μM^{-1}) at varying concentrations of 2-iminopiperidine (**II**). NOS activity was determined by measuring the conversion of L-[2,3-^{3}H]arginine to L-[2,3-^{3}H]citrulline as described in the Experimental Section except that the reaction was initiated by the addition of enzyme and the incubation at 37 °C was for 10 min. Enzyme activity was linear over this time period for each concentration of L-arginine. Each point is the average of duplicate determinations.

in Table 1, compounds **VI** and **VII** are potent inhibitors of each of the human isoforms, with IC_{50} values very similar to that of 2-iminopiperidine. Compound **VIII** was substantially less potent with an IC_{50} of 25 μM for hiNOS.

2-Iminopiperidine (**II**), 2-iminohomopiperidine (**III**), and 2-imino-5,6-dihydro-1,3-thiazine (**VI**) were evaluated in a mouse RAW cell assay to determine their effect on the inhibition of cellular mouse inducible NOS activity. RAW cells were stimulated with lipopolysaccharide (LPS), and the inhibition of nitrite accumulation in the medium was determined. RAW cell mouse inducible NOS IC_{50} values for compounds **II**, **III**, and **VI** were 21, 54, and 14 μM, respectively, demonstrating the ability of these compounds to inhibit the intracellular enzyme.

The ability of 2-iminopiperidine to inhibit inducible NOS *in vivo* was also determined. Oral bioavailability and efficacy were assessed by determining the ability of either 2-iminopiperidine or L-NIL, a previously described inhibitor of inducible NOS, to inhibit the increase in plasma nitrite/nitrate levels generated from inducible NOS following the administration of LPS. Compounds were administered orally by gavage 1 h prior to LPS administration. Plasma nitrite/nitrate levels were measured 5 h following LPS administration and were elevated >15-fold compared to saline-treated animals (428 ± 43 vs 23.2 ± 3.3 μM). Both 2-iminopiperidine and L-NIL inhibited the increase in plasma nitrite/nitrate levels in a dose-dependent manner, demonstrating the ability of both compounds to inhibit inducible NOS activity *in vivo* (Figure 3).

2-Iminopiperidine and the series of cyclic amidines described here represent a new class of potent, non-amino-acid-based NOS inhibitors and the foundation for potential therapeutic agents. Further investigation of this class of inhibitors is in progress.

Experimental Section

L-[2,3-^{3}H]Arginine was purchased from DuPont NEN (Boston, MA); (6*R*)-tetrahydro-L-biopterin was from Research Biochemicals, Inc. (Natick, MA); L-NMA and L-NNA were from

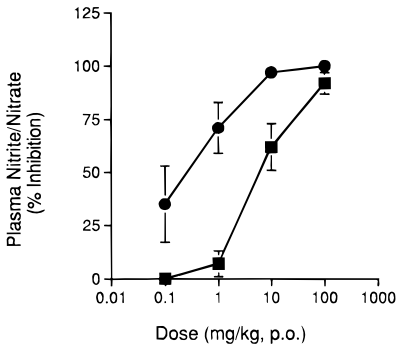


Figure 3. *In vivo* efficacy of 2-iminopiperidine (**II**) and L-NIL in LPS-treated rats. Male Lewis rats were treated with various doses of either 2-iminopiperidine (■) or L-NIL (●) orally 1 h prior to the intraperitoneal administration of 10 mg/kg LPS. Plasma nitrite/nitrate levels were measured 5 h following LPS administration using a fluorometric assay as described in the Experimental Section. Values are means ± SEM for $n = 6$ animals/dose and represent the percent inhibition of animals treated with LPS alone (428 ± 43 μM) compared to saline-treated controls (23.2 ± 3.3 μM).

Sigma (St. Louis, MO); 2-iminopiperidine hydrochloride as well as other chemicals and reagents were purchased from Aldrich Chemical Co. (Milwaukee, WI) or Sigma. L-NIL was prepared and homogeneity assessed as previously described.[13] Purification of synthesized compounds was performed by crystallization or HPLC chromatography on a Waters Prep LC2000 instrument using a Delta Pak C-18 column. ^{1}H- and ^{13}C-NMR spectra were obtained at 300 MHz on a Varian VXR300 spectrometer in D_2O. Mass spectra were obtained on either a VG Model 250 or a Finnigan MAT 90 spectrometer. Elemental analyses were performed by Atlantic Microlabs, Inc. (Norcross, GA). Male Lewis rats (150−200 g) were purchased from Harlan Sprague−Dawley (Indianapolis, IN) and housed and cared for in accordance with the guidelines of the Institutional Animal Care and Use Committee and in accordance with NIH guidelines on laboratory animal welfare. *Escherichia coli* lipopolysaccharide (serotype 0111:B4, Westphal extract) was purchased from Sigma (St. Louis, MO). Ultrafree-MC filter units were from Millipore (Bedford, MA).

Assay of NOS Activity. NOS activity was measured by monitoring the conversion of L-[2,3-^{3}H]arginine to L-[2,3-^{3}H]-citrulline.[13,18] Human inducible NOS, human endothelial constitutive NOS, and human neuronal constitutive NOS were each cloned from RNA extracted from human tissue.[19] The cDNA for hiNOS was isolated from a λcDNA library made from RNA extracted from a colon sample from a patient with ulcerative colitis; the cDNA for hecNOS was isolated from a λcDNA library made from RNA extracted from human unbilical vein endothelial cells (HUVEC); and the cDNA for hncNOS was isolated from a λcDNA library made from RNA extracted from human cerebellum obtained from a cadaver. The recombinant enzymes were expressed in Sf9 insect cells using a baculovirus vector.[19] Enzyme activity was isolated from soluble cell extracts and partially purified by DEAE-Sepharose chromatography.[15,19] The K_m values for L-arginine for hiNOS, hecNOS, and hncNOS were 7, 4, and 6 μM, respectively. To measure NOS activity, 10 μL of enzyme was added to 40 μL of 50 mM Tris (pH 7.6) and the reaction initiated by the addition of 50 μL of a mixture containing 50 mM Tris (pH 7.6), 2.0 mg/mL bovine serum albumin, 2.0 mM DTT, 4.0 mM $CaCl_2$, 20 μM FAD, 100 μM tetrahydrobiopterin, 0.4 mM NADPH, and 60 μM L-arginine containing 0.9 μCi of L-[2,3-^{3}H]arginine. The final concentration of L-arginine in the assay was 30 μM. For hecNOS and hncNOS, calmodulin was included at a final concentration of 40 nM. Following incubation at 37 °C for 15 min, the reaction was terminated by addition of 300 μL of cold buffer containing 10 mM EGTA, 100 mM HEPES (pH 5.5), and 1.0 mM L-citrulline. The [^{3}H]-citrulline was separated by chromatography on Dowex 50W X-8 cation exchange resin and radioactivity quantified with a liquid scintillation counter. All assays were performed at least in duplicate; standard deviations were 10% or less. Production of [^{3}H]citrulline was linear with time for hiNOS, hecNOS, and hncNOS over the time period utilized for the assay.

Measurement of Cellular NOS Activity. The mouse macrophage cell line RAW 264.7 was maintained in Dulbecco's MEM (DMEM) containing 10% fetal bovine serum in 5% CO_2 at 37 °C. Prior to assay, cells were plated at 2×10^5 cells/well onto a 96-well plate in a total volume of 100 μL. The cells were induced to express iNOS with 10 μg/mL LPS in 10% fetal bovine serum containing Earle's salts (without phenol red) and 2 mM L-glutamine for 17 h. Cells were then washed twice with Krebs−Ringer−HEPES (25 mM, pH 7.5) buffer containing 2 mg/mL D-glucose followed by a 1 h preincubation on ice (50 μL total volume) in buffer containing five different concentrations of the inhibitor and 30 μM L-arginine. The assay was initiated by warming the plate to 37 °C. After 120 min, the plate was cooled on ice and 30 μL aliquots were removed for the fluorometric determination of nitrite as previously described.[15,19] The generation of nitrite by the cells was linear for 2 h and the nitrite produced was ca. 100 pmol/well without inhibitor after subtracting out background from uninduced cells. IC_{50} values were the mean of three wells with a SEM of 10−15%.

***In Vivo* Efficacy Determination.** To induce the systemic expression of inducible NOS resulting in markedly elevated (>15-fold) plasma nitrite/nitrate levels,[14,20−22] rats were treated with an intraperitoneal injection of 10 mg/kg LPS. Compounds were administered orally (0.1−100 mg/kg) 1 h prior to LPS administration, and blood was collected 5 h following LPS administration. Plasma was separated and then filtered through a 10 000 MW cutoff Ultrafree microcentrifuge filter unit at 14 000 rpm for 15 min. Plasma nitrite/nitrate concentrations, following conversion of nitrate to nitrite by nitrate reductase, were determined using a fluorometric assay for the measurement of nitrite in biological samples, as described previously.[14,20]

Synthesis of Inhibitors. 2-Iminopyrrolidine Hydrochloride (I). 2-Iminopyrrolidine hydrochloride was prepared by the literature method[23] via reaction of 4-chlorobutyronitrile with ammonia in ethanol in a sealed pressure vessel at 135 °C for 9 h. Filtration of the reaction mixture through Norit, evaporation of solvent, and crystallization from ethanol−ethyl ether afforded the title product in 62% yield as a white solid, mp 149−150 °C. Anal. ($C_4H_9N_2Cl_1 \cdot {}^1/_4H_2O$) C, H, N.

2-Iminohomopiperidine Hydroiodide (III).[24] To a solution of 5 g (0.039 mol) of thiocaprolactam in 100 mL of acetone was added 6.4 g (0.045 mol) of iodomethane. This mixture was stirred for 48 h at 25 °C. Filtration afforded 9.5 g of the thio iminoether hydroiodide salt as a white solid, mp 177−181 °C; 3 g of this iminoether was dissolved in 80 mL of ethanol saturated with anhydrous ammonia, sealed, and stirred at 25 °C for 3 days. Concentration to a reduced volume followed by ethyl ether trituration afforded 2.2 g (39%) of the title product as a white solid mp 135−141 °C. ^{1}H-NMR (D_2O): δ 1.45−1.55 (m, 2H), 1.55−1.7 (m, 4H), 2.5 (m, 2H), 3.28 (m, 2H). MS: 113 (MH^+). Anal. ($C_6H_{13}N_2I_1$) C, H, N.

2-Azacyclooctanone Imine Hydrochloride (IV). A solution of 5 g (0.04 mol) of 2-azacyclooctanone in 15 mL of benzene was stirred at reflux while 4.8 g (0.038 mol) of dimethyl sulfate was added dropwise. After the addition was complete, stirring was continued at reflux for 18 h. The heat was then removed; the reaction mixture was diluted with ethyl acetate and washed with two 100 mL portions of aqueous potassium carbonate. The organic layer was dried ($MgSO_4$), filtered, and concentrated to afford 4.2 g of the corresponding iminoether as a yellow oil; 2.2 g (0.016 mol) of the iminoether was dissolved in 50 mL of anhydrous ethanol, and 0.85 g (0.016 mol) of ammonium chloride was added. This mixture was stirred at 25 °C for 3 days. Removal of the solvent in vacuo afforded 1.7 g (48%) of the title product as a white solid, mp 166−175 °C. ^{1}H-NMR (D_2O): δ 1.3−1.45 (m, 4H), 1.45−1.55 (m, 2H), 1.6−1.7 (m, 2H), 2.45 (m, 2H), 3.3 (m, 2H). MS: 127 (MH^+). Anal. ($C_7H_{15}N_2Cl_1 \cdot {}^1/_4H_2O$) C, H, N.

2-Azacyclononanone Imine Hydrochloride (V). 2-Azacyclononanone imine hydrochloride was prepared analogously to 2-azacyclooctanone imine hydrochloride from 2-azacy-

clononanone to afford 2.75 g (45%) of the 2-azacyclononanone imine as a white solid, mp 108−128 °C. ^{1}H-NMR (D_2O): δ 1.3−1.6 (m, 8H), 1.55−1.65 (m, 2H), 2.45 (m, 2H), 3.35 (m, 2H). MS: 141 (MH^+). Anal. ($C_8H_{17}N_2Cl_1 \cdot {}^1/_3H_2O$) C, H, N.

2-Iminotetrahydropyrimidine Hemisulfate (VIII). 2-Iminotetrahydropyrimidine hemisulfate was prepared by the literature method[25] via reaction of 1,3-diaminopropane with methyl isothiourea hemisulfate in water at reflux for 3 h. Evaporation of solvent and preparative HPLC chromatography (using a linear gradient over 30 min from 0% to 40% acetonitrile in 0.1% trifluoroacetic acid in water) afforded the title product as a white solid in 61% yield, mp 304 °C dec. ^{1}H-NMR (D_2O): δ 1.75 (p, 2H), 3.13 (t, 4H). Anal. ($C_4H_9N_3 \cdot {}^1/_2H_2SO_4$) C, H, N.

2-Imino-5,6-dihydro-1,3-thiazine Hydrochloride (VI).[26] A solution of 4 g (0.03 mol) 3-chloropropylamine hydrochloride and 3 g (0.03 mol) of potassium thiocyanate in 10 mL of water was stirred at 90 °C for 2 h. Evaporation of solvent afforded a semisolid residue which was extracted with boiling ethanol. The title product precipitated from the ethanol upon cooling to afford a white crystalline solid, 2.5 g (55%). Recrystallization from 2-propanol afforded a white solid, mp 110−111 °C. ^{1}H-NMR (D_2O): δ 2.05 (p, 2H), 3.0 (dt, 4H). Anal. ($C_4H_9N_2S_1Cl_1$) C, H, N.

2-Imino-5,6-dihydro-1,3-oxazine Hydrochloride (VII).[27] A solution of 4 g (0.03 mol) of 3-chloropropylamine hydrochloride and 2 g (0.03 mol) of sodium cyanate in 10 mL of water was stirred at 90 °C for 2 h. Evaporation of solvent afforded a semisolid residue which was extracted with boiling ethanol. The title product precipitated from the ethanol upon cooling and addition of ethyl ether to afford a white crystalline solid, 2.5 g (61%), mp 123−124.5 °C. ^{1}H-NMR (D_2O): δ 2.0 (p, 2H), 3.38 (t, 2H), 4.37 (t, 2H).

The picrate salt was prepared, mp 191−201 °C. Anal. ($C_4H_9N_2O_1Cl_1$) C, H, N.

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