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Discovery of a series of aminopiperidines as novel iNOS inhibitors

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Abstract—Nitric oxide (NO), a mediator of various physiological and pathophysiological processes, is synthesized by three isozymes of nitric oxide synthase (NOS). Potential candidate clinical drugs should be devoid of inhibitory activity against endothelial NOS (eNOS), since eNOS plays an important role in maintaining normal blood pressure and flow. A new series of aminopiperidines as potent inhibitors of iNOS were identified from a HTS lead. From this study, we identified compound **33** as a potent iNOS inhibitor, with >25-fold selectivity over eNOS and 16-fold selectivity over nNOS.
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Nitric oxide (NO) is a small reactive molecule with an important role in various physiological processes, including modulation of inflammatory responses and regulation of vessel tone.[1] Nitric oxide synthase (NOS) catalyzes the formation of NO and L-citrulline from L-arginine (Arg) and oxygen. The NOS family consists of three known mammalian isoforms. The neuronal NOS (nNOS) and endothelial NOS (eNOS) are constitutively expressed under non-inflammatory conditions, and their activity is tightly regulated by Ca^{2+}-dependent calmodulin. The third, inducible isoform (iNOS) is a key mediator of inflammation and host defense systems. NO generated by eNOS has been shown to be critical for angiogenesis and for maintaining proper vascular tone. Although there may be pathologies associated with overactivity of eNOS, blood pressure homeostasis is so critical that therapeutically useful NOS inhibitors must not inhibit eNOS. Expression of iNOS is induced at a transcriptional level by inflammatory stimuli, including interferon (IFN), interleukin (IL)-1, tumor necrosis factor (TNF), and bacterial lipopolysaccharide (LPS). Production of excess NO due to the prolonged induction of iNOS has been observed in various inflammatory and autoimmune diseases. Because of the dual nature of NO, the development of selective inhibitors for the inducible NOS isoform is a highly desirable goal. Animal studies suggest that inhibitors of iNOS are useful in treating septic shock, stroke, and many inflammatory diseases including rheumatoid arthritis, osteoarthritis, inflammatory bowel disease and multiple sclerosis.[1–7] Some of the earliest inhibitors of nitric oxide synthase to be reported were direct analogs of the natural substrate L-arginine such as L-NIL (**1**).[8,9] A prodrug of L-NIL, that is, L-N6-(1-iminoethyl)lysine 5-tetrazole amide (SC-51), was evaluated in humans.[10] This clinical study demonstrated that SC-51 produces marked inhibition of exhaled breath NO in normal and asthmatic subjects without producing the side effects observed following the systemic administration of non-selective NOS inhibitors. GW274150 (**2**), a close analog of L-NIL, has also been reported to potently and selectively inhibit iNOS and is currently undergoing Phase II clinical evaluation for the potential treatment of rheumatoid arthritis and migraine.[11,12] Non-amino acid-based inhibitors of iNOS have also been described. In particular, AR-C102222 (**3**), representative of the 1,2-dihydro-4-quinazolinamine series of iNOS inhibitors, demonstrated efficacy in the rat adjuvant-induced arthritis model.[13]

In our search for a novel class of iNOS inhibitors using high throughput screening, we found that the *N*-(5-chloro-2-nitrobenzyl)-*N*,1-dimethylpiperidin-4-amine **4** strongly inhibits human iNOS with an IC_{50} value of 87 nM. Further modification of this lead resulted in

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the discovery of aminopiperidine derivatives as novel iNOS inhibitors. Here, we report the synthesis of these new compounds (Formula A, Fig. 1) and their structure–activity relationships (SARs).

The target compounds **4**–**36**, prepared according to Schemes 1–3, were evaluated for their abilities to inhibit human iNOS, eNOS, and nNOS mediated conversion of [^{3}H]arginine to [^{3}H]citrulline.[14] The potency of these compounds in intact cells was determined by measuring inhibition of cytokine mediated induction of iNOS activity in DLD-1 cells.[15] The screening lead **4**, despite its potent iNOS inhibitory activity, demonstrated no selectivity over the other NOS isoforms (Table 1). In addition, this compound is a nitroaromatic derivative, which is a toxicological concern because of the possible mutagenic and carcinogenic potential of the aromatic nitro group. Many nitroaromatic compounds have been shown to bind covalently to DNA. The reactive forms are metabolically generated through nitro reduction and, in many cases, through oxidative pathways.[16,17] Therefore, one of the objectives of the SAR campaign was to identify a surrogate for the nitro functionality of **4**. Replacement of the 5-chloro-2-nitrobenzyl moiety of **4** with a benzyl (compound **5**), a 2-nitrobenzyl (compound **6**) or a 2-chloro-5-nitrobenzyl (compound **7**) group resulted in a complete loss of the binding toward the iNOS enzyme (Table 1). This demonstrated that the substitution pattern at the benzylic moiety of **4** is of crucial importance for iNOS inhibitory activity. Isosteric

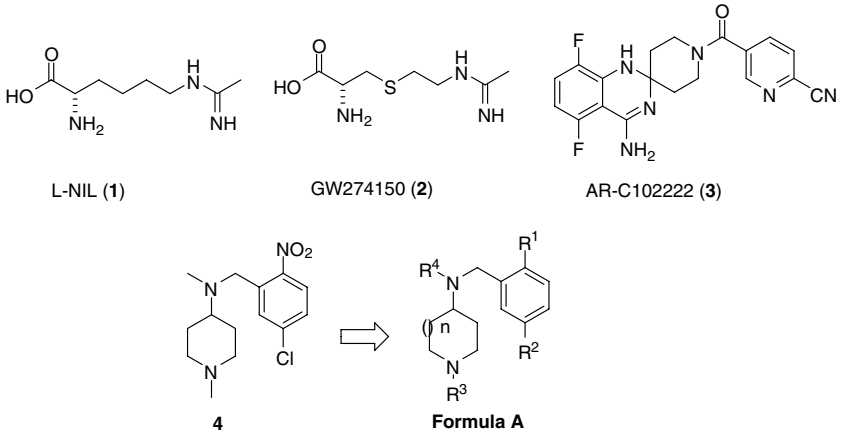
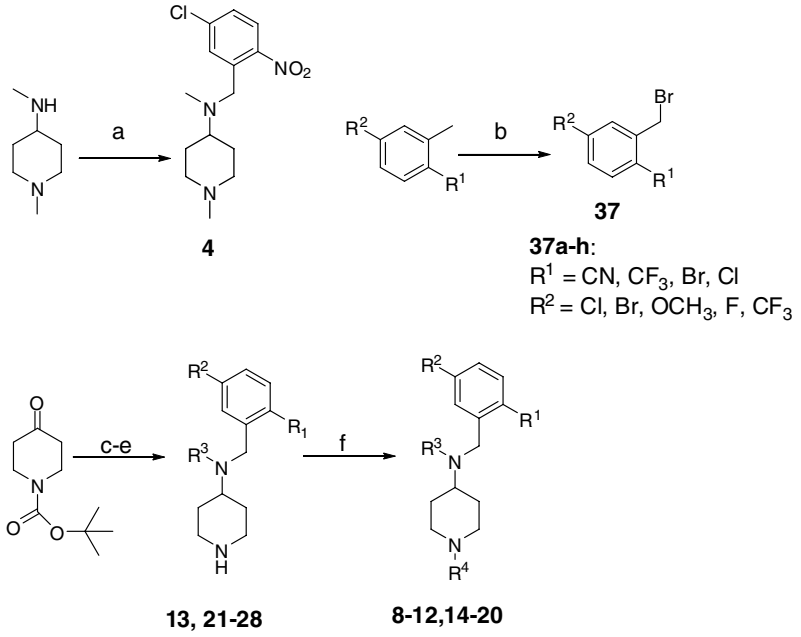
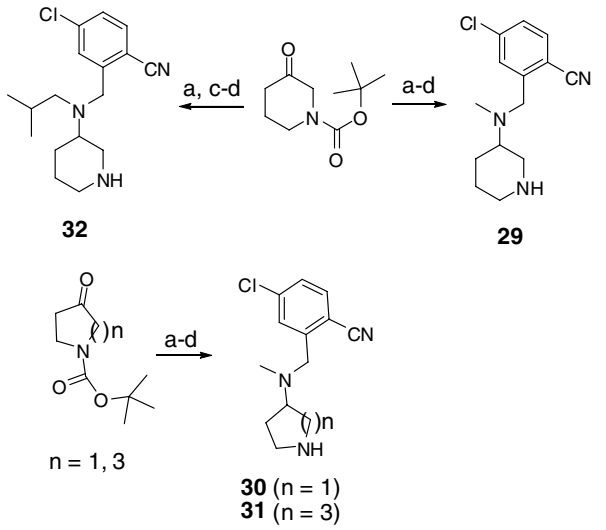


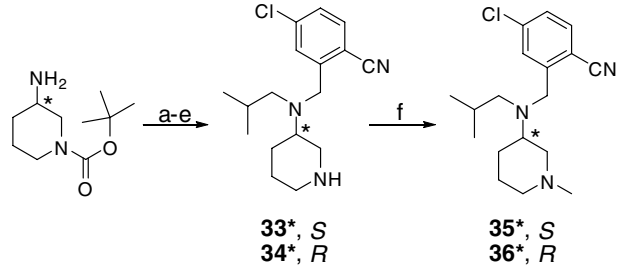
Figure 1. iNOS inhibitors (**1**–**3**), structure of HTS lead (**4**) and general formula of the new compounds prepared (Formula A).



Scheme 1. Reagents and conditions: (a) 5-chloro-2-nitrobenzaldehyde, borane–pyridine complex, EtOH, 25 °C, 32%; (b) NBS, benzoyl peroxide or AIBN, CCl_4, reflux, 36–55%; (c) CH_3COOH, $NaBH(OAc)_3$, R^3NH_2, $ClCH_2CH_2Cl$, 25 °C, 45–100%; (d) **37a**–**h**, K_2CO_3, NaI, DMF or K_2CO_3, acetone, 25 °C, 26–77%; (e) anhyd HCl/Et_2O, MeOH/CH_2Cl_2, 25 °C, or anhyd HCl/dioxane, 25 °C, or CF_3COOH/CH_2Cl_2, 25 °C, or MeOH/H_2O/microwave, 175 °C, 28–100%; (f) Et_3N, CH_3COOH, $NaBH(OAc)_3$, aldehyde, $ClCH_2CH_2Cl$, 25 °C, 32–89%.



Scheme 2. Reagents and conditions: (a) CH_3COOH, $NaBH(OAc)_3$, $C_6H_5CH_2NHCH_3$ or $(CH_3)_2CHCH_2NH_2$, CH_2Cl_2, 25 °C, 68–86%; (b) H_2, Pd/C, EtOH, 25 °C, 78–94%; (c) 2-bromomethyl-4-chloro-benzonitrile, K_2CO_3, NaI, DMF, 25 °C, 64–91%; (d) anhyd HCl/dioxane, 25 °C, 90–97%.

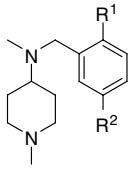


Scheme 3. Reagents and conditions: (a) 2,4-dinitrobenzenesulfonyl chloride, 2,6-lutidine, CH_2Cl_2, THF, 25 °C, 60–81%; (b) $(CH_3)_2CHCH_2OH$, PPh_3, DEAD, THF, 25 °C, 80–90%; (c) $CH_3(CH_2)_3NH_2$, CH_2Cl_2, 25 °C, 90%; (d) 2-bromomethyl-4-chloro-benzonitrile, K_2CO_3, NaI, DMF, 25 °C, 50–69%; (e) anhyd HCl/dioxane, 25 °C, 92–99%; (f) formaldehyde, CH_3COOH, $NaBH(OAc)_3$, CH_2Cl_2, 25 °C, 82–87%.

replacement of the nitro functionality of **4** with a cyano group (compound **8**), which retained electron-withdrawing and hydrogen-bond acceptor properties, resulted in a 3-fold decrease in the potency for the iNOS enzyme and a 4-fold decrease in the cellular iNOS inhibitory activity. Additional benzylic substitutions were explored (i.e., **9**–**12**) but these modifications did not result in any improvement in potency for iNOS. SAR at the piperidine nitrogen of **8** was then investigated (Table 2). The unsubstituted derivative **13**inhibited iNOS with a potency comparable (IC_{50} = 330 nM) to its *N*-methyl analog (compound **8**; IC_{50} = 290 nM). Replacement of the *N*-methyl group of **12** with ethyl, propyl, butyl, isobutyl or cyclopropylmethyl substituents (compounds **14**–**18**, respectively) was found to be well tolerated. However, these modifications did not result in a significant improvement in the potency for iNOS, selectivity or cellular iNOS inhibitory activity. The *N*-benzyl derivative **19**, however, retained good iNOS potency (IC_{50} = 270 nM) and displayed a better selectivity profile over eNOS (16-fold) and nNOS (7-fold) when compared to its *N*-methyl analog **8** (3-fold selective over eNOS; 2-fold selective over nNOS). In contrast, compound **20**, the *N*-phenethyl analog of **19**, did not show any selectivity over the other iNOS isoforms.

Further, modification of compound **13** was explored to increase the potency and selectivity for iNOS (Table 3). Replacement of the *N*-methyl group of **13** with an *N*-ethyl functionality (compound **21**) resulted in a 3-fold increase in potency for iNOS. Furthermore, the selectivity profile of **21** was improved when compared to the selectivity profile of **13**. In addition, compound **21** was found to be 3-fold more potent than its *N*-methyl analog in the whole cell assay. This finding suggested that introduction of an appropriately sized hydrophobic alkyl group at the R^4-position of compounds of Formula A (Fig. 1) would enhance the potency for iNOS, eNOS, and nNOS selectivity, and potency in the iNOS cellular assay. Substitution of the *N*-ethyl group of **21** with an *N*-propyl moiety (compound **22**) resulted in an increase in potency in the cellular assay. This might be attributed to a concurrent increase in the overall lipophilicity of the molecule resulting in improved cell membrane permeability. Further extension of this key alkyl chain to a butyl moiety (compound **23**) led to a significant decrease in the potency for iNOS as well as potency in the cellular assay. The *N*-isopropyl analog **24** showed strong inhibitory activity against iNOS. This derivative, which was the most active compound in this study, also displayed moderate selectivity over the eNOS (18-fold) and nNOS (6-fold) isoforms. In order to explore the size of the lipophilic pocket in which the isopropyl of **24** is thought to interact (see molecular modeling section), we prepared the *N*-isobutyl (compound **25**), *N*-cyclopropyl methyl (compound **26**), *N*-cyclobutyl (compound **27**), and *N*-phenyl (compound **28**) analogs of **24**. These structural modifications led to a decrease in the potency for the iNOS enzyme, indicating that the hydrophobic cavity in which the isopropyl group of **24** interacts is relatively small. The *N*-isobutyl derivative **25**, which retained good potency for iNOS (IC_{50} = 390 nM) and cellular activity (IC_{50} = 570 nM), was nonetheless of special interest for further optimization, since this compound interacts weakly with the eNOS isoform even at high concentrations (20% inhibition at 10 μM). As indicated in Table 4, the 3-substituted piperidine analog **29** also displayed good potency for iNOS (IC_{50} = 180 nM). In contrast, substitution of the piperidine template of **13** with a pyrrolidine (compound **30**) or azepine (compound **31**) scaffold resulted in a significant decrease in iNOS potency. Based on these results, we prepared the 3-substituted piperidine analog of **25**, that is, compound **32**, and found that this compound retained good potency for iNOS and displayed high selectivity against eNOS (15% inhibition at 10 μM). Since **32** was racemic, the synthesis of enantiomers **33** and **34** was undertaken to see if additional potency/selectivity were to be gained. As indicated in Table 4, the *S*-isomer **33** was superior to the *R*-isomer **34**, in terms of iNOS cellular activity and selectivity over eNOS and nNOS. Compound **35**,

Table 1. NOS inhibitory activity and selectivity of compounds **1**–**12**



Compound	R^1	R^2	IC_{50}[a] (nM)			Selectivity[b]	Selectivity [c]	iNOS cellular assay
			iNOS	eNOS	nNOS	eNOS/iNOS	nNOS/iNOS	IC_{50}[d] (nM) or % inhibition at 10 μM
4	NO_2	Cl	90	180	80	2	1	1100
5	H	H	ns[f]	nd[e]	nd[e]	nd[e]	nd[e]	ns[f]
6	NO_2	H	ns[f]	nd[e]	nd[e]	nd[e]	nd[e]	ns[f]
7	Cl	NO_2	ns[f]	nd[e]	nd[e]	nd[e]	nd[e]	ns[f]
8	CN	Cl	290	770	540	3	2	3700
9	CN	Br	210	810	530	4	3	3700
10	CN	OCH_3	5100	nd[e]	nd[e]	nd[e]	nd[e]	ns[f]
11	CF_3	Cl	700	1400	780	2	1	48%
12	Br	Cl	420	820	590	2	1	51%
1 (L-NIL)			280	2500	6000	9	21	31,000
2 (GW274150)			220	ns[f]	8400	>45	38	26%
3 (AR-C102222)			170	ns[f]	840	>70	5	210

[a] Inhibition of human iNOS, eNOS, and nNOS mediated conversion of [^{3}H]arginine to [^{3}H]citrulline by tested compounds. IC_{50} values were determined by testing each compound at eight concentrations. IC_{50} values are geometric means computed from at least three separate determinations.

[b] Selectivity was defined as the ratio of IC_{50} value of eNOS to that of iNOS.

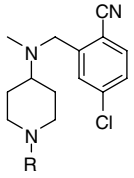
[c] Selectivity was defined as the ratio of IC_{50} value of nNOS to that of iNOS.

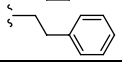
[d] Inhibition of cytokine mediated induction of iNOS activity in DLD-1 cells by tested compounds. IC_{50} values were determined by testing each compound at eight concentrations. IC_{50} values are geometric means computed from at least three separate determinations.

[e] nd, not determined.

[f] ns, no significant effect (<15% inhibition at 10 μM).

Table 2. NOS inhibitory activity and selectivity of compounds **13**–**20**



Compound	R	IC_{50}[a] (nM)			Selectivity[b]	Selectivity[c]	iNOS cellular assay
		iNOS	eNOS	nNOS	eNOS/iNOS	nNOS/iNOS	IC_{50}[d] (nM)
13		330	1100	350	3	1	5900
14		370	450	370	1	1	4500
15		670	1100	810	2	1	4400
16		340	680	420	2	1	3800
17		400	540	600	1	2	3200
18		250	860	530	3	2	3500
19		270	4300	1800	16	7	3500
20		300	220	150	1	0.5	3300

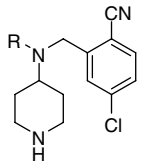
[a] Inhibition of human iNOS, eNOS, and nNOS mediated conversion of [^{3}H]arginine to [^{3}H]citrulline by tested compounds. IC_{50} values were determined by testing each compound at eight concentrations. IC_{50} values are geometric means computed from at least three separate determinations.

[b] Selectivity was defined as the ratio of IC_{50} value of eNOS to that of iNOS.

[c] Selectivity was defined as the ratio of IC_{50} value of nNOS to that of iNOS.

[d] Inhibition of cytokine mediated induction of iNOS activity in DLD-1 cells by tested compounds. IC_{50} values were determined by testing each compound at eight concentrations. IC_{50} values are geometric means computed from at least three separate determinations.

Table 3. NOS inhibitory activity and selectivity of compounds **21**–**28**



Compound	R	IC_{50}[a] (nM) or % inhibition at 10 μM			Selectivity[b]	Selectivity[c]	iNOS cellular assay
		iNOS	eNOS	nNOS	eNOS/iNOS	nNOS/iNOS	IC_{50}[d] (nM)
21		100	1000	360	10	4	580
22		130	1700	140	13	1	280
23		570	nd[e]	nd[e]	nd[e]	nd[e]	5000
24		32	570	190	18	6	380
25		390	20%	1900	>25	5	570
26		160	2100	748	13	5	1400
27		1500	1%	24%	>7	>6	6700
28		660	0%	33%	>15	>15	8000

[a] Inhibition of human iNOS, eNOS, and nNOS mediated conversion of [^{3}H]arginine to [^{3}H]citrulline by tested compounds. IC_{50} values were determined by testing each compound at eight concentrations. IC_{50} values are geometric means computed from at least three separate determinations.

[b] Selectivity was defined as the ratio of IC_{50} value of eNOS to that of iNOS.

[c] Selectivity was defined as the ratio of IC_{50} value of nNOS to that of iNOS.

[d] Inhibition of cytokine mediated induction of iNOS activity in DLD-1 cells by tested compounds. IC_{50} values were determined by testing each compound at eight concentrations. IC_{50} values are geometric means computed from at least three separate determinations.

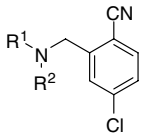
[e] nd, not determined.

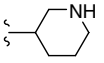
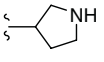
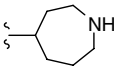
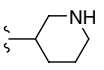
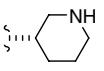
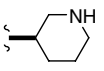
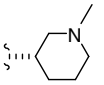
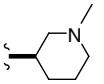
the *N*-methylated analog of **33**, resulted in an improvement in cellular potency, while the potency and selectivity for nNOS was reduced when compared to **33**.

Several crystal structures of various ligands co-crystallized with the mouse or human iNOS dimer have been published.[18–24] The active form of the enzyme is a homodimer with two independent active sites, each containing a heme group and a tetrahydrobiopterin cofactor. Each L-arginine binding site is located at the interface between the two monomers and is comprised of residues from both monomers. An extensive network of hydrogen bonds between L-arginine, the carboxylate sidechain of Glu377 (human iNOS numbering), the backbone amide of Met374, the backbone carbonyl of Trp372, the propionate tails of the heme, and the pterin co-factor stabilizes the active site structure when L-arginine is bound.[20] Examination of the crystal structures of various ligands which bind competitively with L-arginine reveals two distinct classes. One class mimics a critical bidentate interaction between the guanidinium group of arginines and the carboxylate of Glu377 in the iNOS active site. A second class of ligands, without the ability to form this bidentate interaction, is stabilized in the iNOS active site by π-stacking with the heme group.[20] The induced-fit docking protocol of the Schrödinger suite[24] was used to dock **13**, a presumably π-stacking ligand, into the iNOS active site. As shown in Figure 2a and b, there are several key interactions between the inhibitor and the enzyme: (1) the cyano group of **13** forms a hydrogen-bond with the amide hydrogen of Met374; (2) the basic nitrogen of the piperidine ring, protonated at physiological pH, forms two hydrogen bonds with the iNOS active site, one with Asp382 and another with one of the propionate tails of the heme. These interactions are consistent with those seen in crystal structures of other π-stacking iNOS ligands; (3) the methyl group of **13** interacts with a small hydrophobic pocket of the active site; and (4) the cyano phenyl moiety of **13** interacts by π stacking with the heme.

Electron-withdrawing substituents on the benzylic ring are essential for the inhibitor to form a strong π-stacking interaction with the heme. Removal of either electron-withdrawing substituent from the ring disrupts the π-stacking interaction by making the benzylic π-system a poorer acceptor. The docking pose of **13** high-

Table 4. NOS inhibitory activity and selectivity of compounds **29–36**



Compound	R^1	R^2	IC_{50}[a] (nM) or % inhibition at 10 μM			Selectivity[b]	Selectivity [c]	iNOS cellular assay
			iNOS	eNOS	nNOS	eNOS/iNOS	nNOS/iNOS	IC_{50}[d] (nM) or % inhibition at 10 μM
29			180	983	190	5	1	4400
30			1200	4000	1200	3	1	33%
31			660	4400	1200	7	2	31%
32			290	15%	2500	>15	9	1200
33			330	0%	5300	>25	16	570
34			260	23%	1200	>25	5	1500
35			720	0%	6500	>50	9	320
36			470	0%	4900	>15	10	660

[a] Inhibition of human iNOS, eNOS, and nNOS mediated conversion of [^{3}H]arginine to [^{3}H]citrulline by tested compounds. IC_{50} values were determined by testing each compound at eight concentrations. IC_{50} values are geometric means computed from at least three separate determinations.

[b] Selectivity was defined as the ratio of IC_{50} value of eNOS to that of iNOS.

[c] Selectivity was defined as the ratio of IC_{50} value of nNOS to that of iNOS.

[d] Inhibition of cytokine mediated induction of iNOS activity in DLD-1 cells by tested compounds. IC_{50} values were determined by testing each compound at eight concentrations. IC_{50} values are geometric means computed from at least three separate determinations.

lights the need for a hydrogen-bond acceptor at the cyano-position on the benzylic ring (position R^1 of Formula A). The cyano group is oriented toward Met374 and this hydrogen-bond interaction is important for the stability of the ligand–enzyme interaction. Additionally, the *N*-methyl group at the R^4-position projects into a small hydrophobic pocket of the enzyme active site. Slightly larger R^4 substituents (ethyl, propyl, isopropyl, cyclopropylmethyl) improve binding by increasing favorable hydrophobic interactions with the enzyme. Bulkier substituents, however, encounter steric restrictions in this pocket which reduce the ligand affinity for the enzyme.

Screening of the Adolor compound collection provided a novel lead, *N*-(5-chloro-2-nitrobenzyl)-*N*,1-dimethylpiperidin-4-amine **4**, displaying strong iNOS inhibitory activity but no selectivity over the other NOS isoforms. Further modification of this lead resulted in the discovery of aminopiperidines as a novel chemical class of iNOS inhibitors. The substitution pattern in the phenyl ring of these classes of compounds was found to be of key importance for iNOS inhibitory activity. In addition, introduction of an isopropyl group at the R^4-position was critical to disrupt the interaction of the compounds with eNOS while maintaining potent iNOS inhibitory activity in the enzyme assay as well as in the cellular assay. This work ultimately led to the identification of compound **33**, which displayed better activity in the iNOS whole cell assay and a much improved selectivity profile over eNOS and nNOS when compared to the original lead.

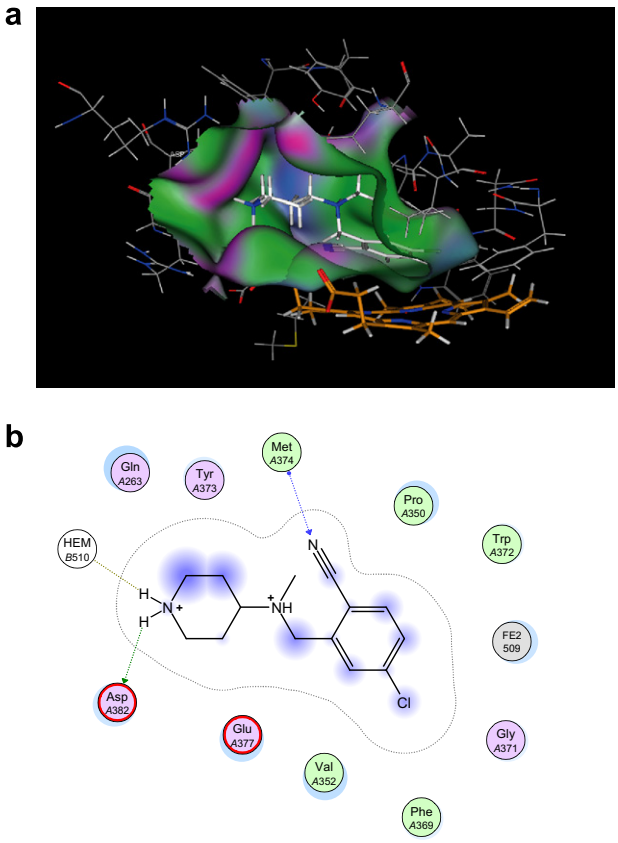


Figure 2. (a) Compound **13** (white) docked into the iNOS active site. The aromatic ring exhibits π-stacking with the heme (orange). On the surface, green represents hydrophobic regions, blue represents mildly polar regions of the active site, and pink shows hydrogen-bonding areas. The large green area surrounding the central amino group underlies the enzyme's preference for lipophilic substituents on the amino nitrogen. Pink regions near the hydrogens on the piperidine nitrogen highlight H-bond interactions of the ligand with one of the heme propionate tails and Asp382. The pink region near the cyano nitrogen indicates the presence of an H-bond interaction with the amide nitrogen of Met374. (b) H-bond interactions with Asp382, Met374, and the heme propionate tail are shown in the ligand interaction diagram. Blue regions around ligand atoms indicate the degree of solvent exposure.

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14. In vitro method: human iNOS, nNOS, and eNOS mediated conversion of [^{3}H]arginine to [^{3}H]citrulline. Compounds were evaluated for their abilities to inhibit human iNOS (human iNOS endogenously expressed in colon adenocarcinoma cells (DLD-1 cells)), eNOS (human recombinant eNOS expressed in SF9 cells), and nNOS (human recombinant eNOS expressed in SF9 cells) mediated conversion of [^{3}H]arginine to [^{3}H]citrulline. Series of concentrations of compounds were incubated with 10 nM [^{3}H]arginine, [^{14}C]citrulline (80–120 nM) as an internal standard, cofactors (1.4 mM β-NADP$^+$, 3.0 mM glucose-6-phosphate (G6P), 3.4 mM $MgCl_2$, 0.4 U/mL G6P dehydrogenase, 5.0 μM FAD, 5.0 μM FMN, 5.0 μM BH_4), and enzyme in 100 μL of 50 mM Hepes buffer for 1 h at 37 °C. For eNOS and nNOS assays, additional cofactors 25 nM calmodulin, 0.5 mM $CaCl_2$, and 3.5 mM glutathione were included in the reactions. The reactions were stopped by the addition of 25 μL of 1 M MES and filtered through AG-50W-X8 (200–400 mesh, Na form) cation exchange resin that had been loaded onto 96-well filter plates using a 100 μL column loader (Millipore). The filters were then washed serially with 75 μL and 25 μL of ddH_2O, which was collected in the collection plate, and the radioactivity in the filtrate counted. Background activity was determined in the presence of 10 μM AMT. Data were fit to the one-site competition model in GraphPad Prism (version 4.02 for Windows) and are from a single experiment that was repeated at least four times with similar results.
15. In vitro method: cytokine mediated induction of iNOS in DLD-1 cells. Human colon adenocarcinoma (DLD-1) cells, obtained from the American Type Culture Collection (ATCC), were plated into 96-well plates at 40,000 cells per well 3 days before the experiment. On the day of the experiment, the medium was removed and replaced with 90 μL of fresh complete growth medium containing 20 ng/mL interferon γ (INFγ), 4 ng/mL tumor necrosis factor α (TNFα), 2 ng/mL interleukin 1β (IL1β), and 1 mM L-arginine. Background iNOS activity was determined using complete growth medium in the absence of cytokines and L-arginine. Titrations of test compounds (10 μL) in 50 mM Hepes, pH 7.4, were then added to the appropriate wells and the assay incubated for 24 h at 37 °C with 5% CO_2. After incubation, an aliquot from each well (75 μL) was transferred to a clear, untreated 96-well plate containing an equal volume of modified Griess reagent (Sigma). The absorbance at 540 nm was measured for each well using the Fusion (Perkin-Elmer Life Sciences). IC_{50} values were determined from non-linear regression fits of the data in GraphPad Prism (version 4.02 for Windows).
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