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1-(1,3-Benzodioxol-5-ylmethyl)-3-[4-(1*H*-imidazol-1-yl)phenoxy]-piperidine analogs as potent and selective inhibitors of nitric oxide formation

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Abstract—A new series of 1-(1,3-benzodioxol-5-ylmethyl)-3-[4-(1*H*-Imidazol-1-yl)phenoxy]-piperidine analogs were designed and identified as potent and selective inhibitors of NO formation based both on the crystal structure of a murine iNOS Δ114 monomer domain/ inhibitor complex and inhibition of the NO formation in human A172 cell assays. Compound **12S** showed high potency and high iNOS selectivity versus nNOS and eNOS.

Nitric oxide (NO) is generated from the reaction of L-arginine with oxygen by an enzyme called nitric oxide synthase (NOS).[1,2] NOS exists in three distinct isoforms, which fall into two categories: constitutive and inducible. There are two constitutive isoforms, which are calcium and calmodulin dependent, and there is an inducible isoform, which is calcium independent. The two constitutive isoforms are (1) a neuronal isoform, NOS-1 or nNOS,[3] which was first found in neuronal tissue and (2) an endothelial isoform, NOS-3 or eNOS, which is found in vascular endothelial cells. The inducible isoform, NOS-2 or iNOS, is found in a wide range of cells. The three isoforms differ in their location and function, but are similar in that they are only active in the dimeric form. eNOS and nNOS generate low, transient levels of NO in response to an increase in intracellular calcium concentrations to regulate blood pressure, platelet adhesion, and neurotransmission.[4] However, iNOS generates high, sustained levels of NO. These elevated levels of NO and resulting NO-derived metabolites result in cellular cytotoxicity and tissue damage which may contribute to the pathophysiology of a number of human diseases such as multiple sclerosis (MS) and rheumatoid arthritis (RA). The non-selective inhibition of NO formation might lead to unwanted side effects, therefore much effort has been focused on the discovery of novel selective iNOS inhibitors in the pharmaceutical industry.[4,5]

The search for direct enzyme inhibitors of iNOS led to the discovery of *N*-phenylimidazoles as selective inhibitors of iNOS, albeit with moderate potency.[6] In addition, *N*-phenylimidazoles can also promote iNOS dimer assembly[5d] after binding to the heme. Because the dimer assembly is critical for NOS activity and NO production, prevention of iNOS dimerization is a potential mechanism for a therapeutic agent.

We have previously disclosed a class of compounds that block iNOS dimerization.[5a,b] A representative of that class is **1** (Fig. 1). A comparison of this compound

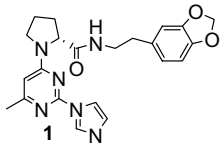

Figure 1. iNOS dimerization inhibitor.

Keywords: iNOS inhibitor; Dimerization.
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bound to the murine iNOS Δ114 monomer domain and the model of the iNOS dimer suggests that these inhibitors occupy the same position as Glu377 in the human iNOS dimer structure. Our working hypothesis is that these inhibitors bind to the iNOS monomer and prevent Glu377 of helix 7A from occupying the position that leads to dimer formation.

The original series contain an imidazol-1-ylpyrimidine linked to a distal benzodioxolane group with a six-atom tether including a saturated heterocycle. From the crystal structure of compound **1** bound to monomeric murine iNOS Δ114 (Fig. 2), we knew that the imidazole group binds to the heme and the imidazole, pyrimidine, and pyrrolidine ring are nearly coplanar. The benzodioxolane group fits snugly between residues in the iNOS monomer active site and the pyrimidine ring resulting in a U-shaped conformation. This binding mode is consistent with the crystal structure of the fragments, 4-imidazol-1-ylphenol and piperonylamine bound, to murine iNOSΔ114 (not shown).[7] This prompted us to design new inhibitors with alternate linkers connecting the benzodioxolane and imidazole moieties (Fig. 3). Here we report the discovery of potent, selective inhibitors of NO formation, which do not inhibit iNOS enzyme activity.

Our initial work focused on compounds with a hydroxyethylamine as a linker (Scheme 1). Reductive amination

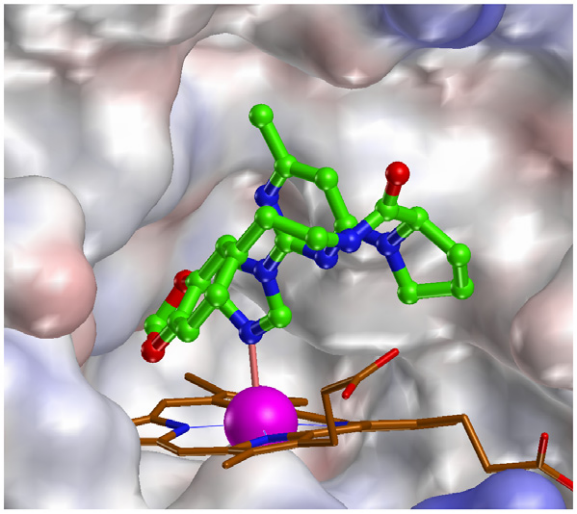


Figure 2. The crystal structure of inhibitor **1** bound to murine iNOS monomer (PDB entry 2ORO).

of 2-[4-(imidazol-1-yl)phenoxy]ethylamine **2** with piperonal gave monoalkylated compound **3** and dialkylated compound **4**. Alkylation of **3** with ethyl bromoacetate under basic conditions, followed by hydrolysis with lithium hydroxide, afforded compounds **5** and **6**, respectively. The acid, **6**, was bound to an oxime resin using DCC as coupling reagent, followed by reaction with amines, affording products **7**–**9**. The heterocyclic compounds were prepared by standard methods (Scheme 2). The reaction of 3-hydroxypiperidine hydrochloride **10** with 3,4-methylenedioxybenzyl chloride using potassium carbonate at 50 °C gave the alkylated intermediate **11**. Mitsunobu reaction[8] of **11** with 4-(imidazol-1-yl)phenol afforded **12**. The other phenoxy analogs were prepared in a similar fashion. Oxidation of *N*-benzyl-3-hydroxypiperidine hydrochloride **13** with PDC followed by reductive amination with 4-(*N*-imidazol-1-yl)aniline afforded intermediate **14**. Further debenzylation of **14** with Pd/C followed by alkylation with 3-4-methylenedioxybenzyl chloride gave **15**. For the preparation of **17**, compound **13** was treated with sodium hydride followed by reaction with 3-chloro-6-(imidazol-1-yl)pyridazine to give **16**. Debenzylation and alkylation of **16** afforded **17**.

Compounds were initially tested for inhibition of NO formation in a whole cell using human A172 cell assays induced with cytokines.[5c] NO formation was monitored with Griess reagent.[9] In a standard iNOS enzyme assay, compounds had weak activity (compound **12** had an IC_{50} of 15.5 μM). Selectivity between the NOS isoforms was determined in SF9 cells transfected with a NOS isoform in a tetracycline-induced system.[5c] NOS activity was monitored by measuring the production of radiolabeled citrulline from radiolabeled arginine. Tet data refer to the IC_{50} determined as an average of at least two separate experiments in the iNOS transfected cells induced by addition of tetracycline unless otherwise stated.

We first explored straight chain tethered analogs (Table 1). The initial compound **3**, with an unsubstituted linker, had surprising activity, with an IC_{50} of 240 nM, but substitution of the chain was detrimental, although attempts were limited. Tertiary amines were tolerated, but compound **6** with an acetic acid substitution showed weak activity.

Next, we explored the SAR of analogs with a heterocyclic linker (Table 2). Compound **18**, with pyrrolidine as a linker, exhibited low activity with an IC_{50} of 4 μM.

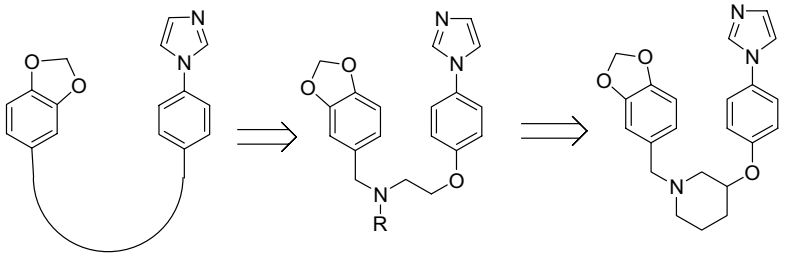
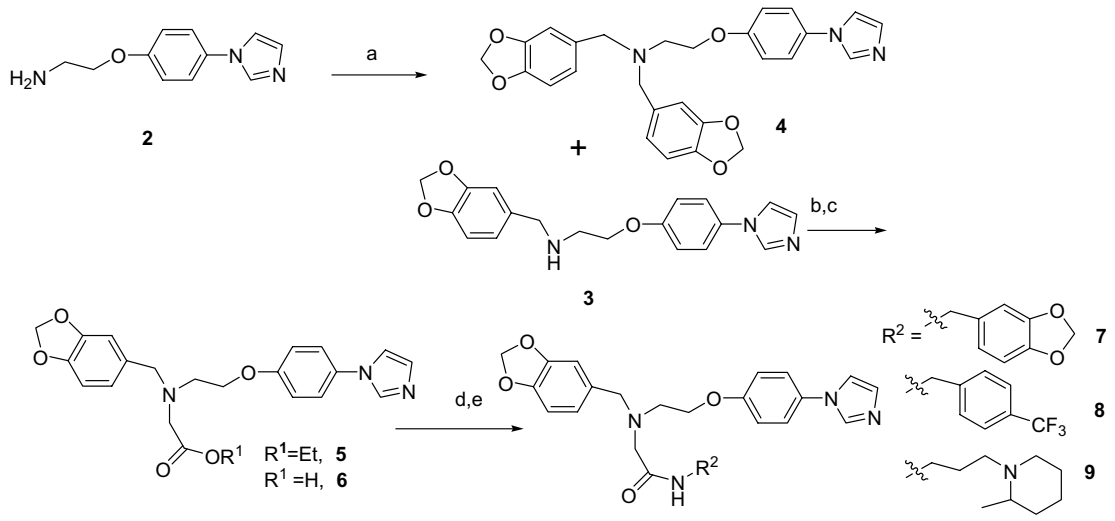
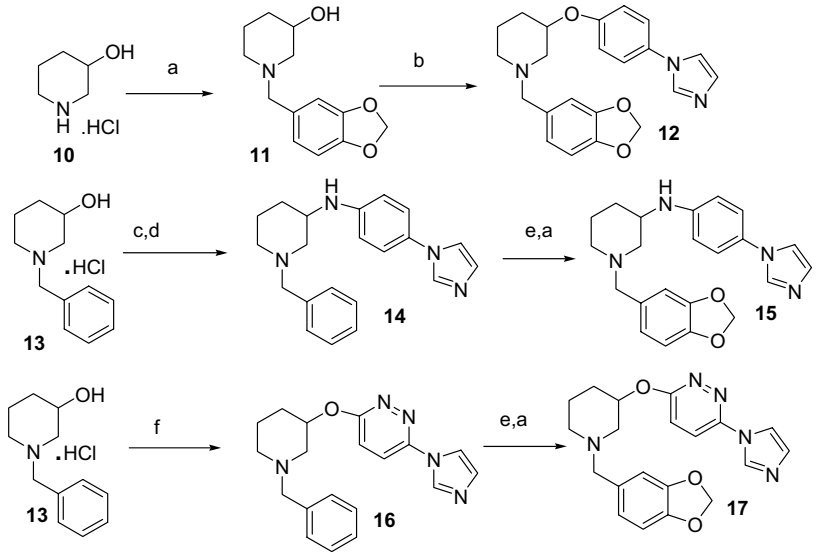


Figure 3. iNOS dimerization inhibitor design scheme.



Scheme 1. Reagents and conditions: (a) $NaBH_3CN$, AcOH, MeOH, piperonal; (b) ethyl bromoacetate, K_2CO_3, MeCN, 70–80 °C, 3 h; (c) LiOH, THF–H_2O; (d) DCC, DCM, Kaiser oxime resin, 18 h; (e) NH_2R^2, DCM 18 h.



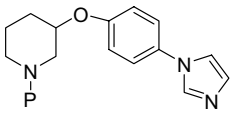
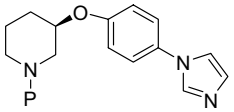
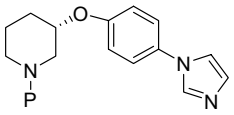
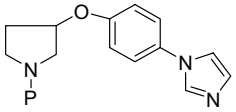
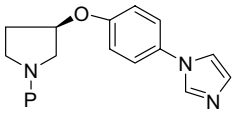
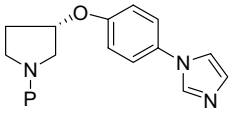
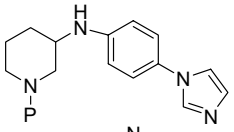
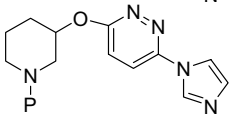
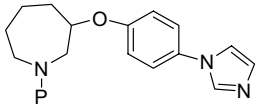
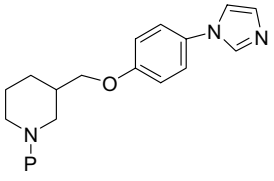
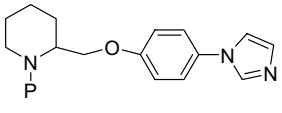
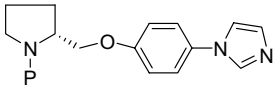
Scheme 2. Reagents and conditions: (a) 3-4-methylenedioxybenzyl chloride, K_2CO_3, NaI (cat.), DMF, 50 °C, 4 h; (b) 4-1-imidazolylphenol, Ph_3P, DEAD, DMF; (c) PDC, DCM; (d) 4-(*N*-imidazolyl)aniline, $Ti(OiPr)_4$, $NaCNBH_3$, EtOH, 20 h; (e) 10% Pd/C, MeOH, NH_4CO_2H, reflux, 4 h; (f) i—NaH, DMF; ii—3-chloro-6-(imidazol-1-yl) pyridazine, rt.

Table 1. Inhibitory activity for compounds **3**–**9**

Compound	A172 cell IC_{50} (μM)
3	0.24
4	1.5
5	1.2
6	>10
7	0.64
8	1.8
9	1.6

The activity resided in the S enantiomer, as **18R** displayed weaker activity ($IC_{50} > 10$ μM). Changing the linker attachment site to the 2-position with one more carbon extension gave **22R**, which exhibited 5-fold higher potency and showed some selectivity (>80-fold for both). When the pyrrolidine was replaced with a piperidine, **12**, a highly potent and selective compound was obtained. Again, the activity resided in the S enantiomer, as **12R** displayed weaker activity. Replacement of the imidazol-1-ylphenyl group with a 6-(imidazol-1-yl)-3-pyridazinyl group, **17**, resulted in similar activity and selectivity. Compound **15**, an aniline analog, showed 5-fold higher potency than the ether **12**. Extension by one more carbon off the piperidine (**20**) significantly reduced the potency. Compound **19** with an azepine as the linker showed similar activity to the piperidine analog **12**. The 2-(4-imidazol-1-yl)phenoxy methyl substituted analog **21** exhibited 2-fold more potency than the pyrrolidine analog **22R**. The 3S-hydroxypiperidine was optimal as a linker and compound **12S** was optimal both in high potency with IC_{50} value of 9 nM in a whole cell assay and in iNOS high selectivity versus nNOS and eNOS (>400 for both). It should be noted that the differ-

Table 2. Activity and selectivity of piperidine analogs

Compound	Structure	A172 cell IC_{50} (nM)	Tet iNOS IC_{50} (nM)	Tet selectivity ratio n/i	Tet selectivity ratio e/i
12		57	250	350	400
12R		1700	NT	NT	NT
12S		9	72	600	420
18		4200	NT	NT	NT
18R		>10,000	NT	NT	NT
18S		1600	NT	NT	NT
15		10	120	439	86[a]
17		52	590	>17	>17
19		78	NT	NT	NT
20		>10,000	NT	NT	NT
21		100	2200	>46	>46
22R		250	1200	>80	>80[a]

P = 3,4-methylenedioxybenzyl group. NT, not tested.
[a] Data were reported as for $n = 1$ determination.

ences in potency between the A172 cells and the tetracycline-induced cell assays are unclear, although it may be due to differences in NO production between the different cell types.

Further characterization of **12S** was pursued due to its activity and selectivity (Table 3). Compound **12S** exhibited a high clearance in the rat and extensive distribution throughout the body. Oral bioavailability is high partly because of the low systemic level after intravenous administration. Metabolic stability in rat, dog, and human liver microsomes revealed a 47%, 89%, and 31% parent remaining after 3 h, respectively.

Table 3. Rat PK data of compound **12S**

Route	iv	po
Dose (mg/kg)	2	10
$T_{1/2}$ (h)	2.2	2.9
Cl (ml/min/kg)	128	na
V_{ss} (L/kg)	21	na
C_{max} (μg/ml)	na	0.8
AUC_{inf} (h μg/ml)	0.28	2.6
%*F*	na	~100%

na, not applicable.

These results possibly account for the high clearance.

We determined the crystal structure of **12S** bound to the murine iNOSΔ 114 monomer (Fig. 4). The compound occupies the arginine binding pocket and the imidazole directly coordinates to the iron. The benzodioxolane and phenylimidazole adopt a favorable U-shaped conformation, positioning the benzodioxolane such that it will prevent Glu377 in helix 7A from adopting a conformation that is conducive to dimer formation, an essential step for activity.

A comparison between the crystal structures of **1** and **12S** bound to murine iNOS Δ114 monomer shows that both compounds bind in a U-shaped conformation, and that the 4-(imidazol-1-yl)aryl binds in the same conformation, with the imidazole coordinating the heme (Fig. 5). The benzodioxolane moieties occupy the same pocket between the arylimidazole and the iNOS active site, but have different orientations. Compound **12S** is 20-fold less potent than **1**. This difference of potency could be due to the extra interactions that the imidazolylpyrimidine of **1** has with iNOS and the heme (methyl group with Pro344 and Gln257 and pyrrolidine ring with heme) and/or the different orientations of the benzodioxolane groups.

Replacement of the phenoxy oxygen atom with a nitrogen atom shown in structure **15** presumably does not change the U-shaped conformation which accounts for the similar potency to compound **12**. However, when the linker was changed from piperidine (**12S**) to pyrrolidine (**18S**) the potency drops 178-fold. A model of **18S** in iNOS monomer shows that the pyrrolidine ring needs to adapt a strained conformation in order to position the phenylimidazole and benzodioxolane moieties in a productive binding conformation.

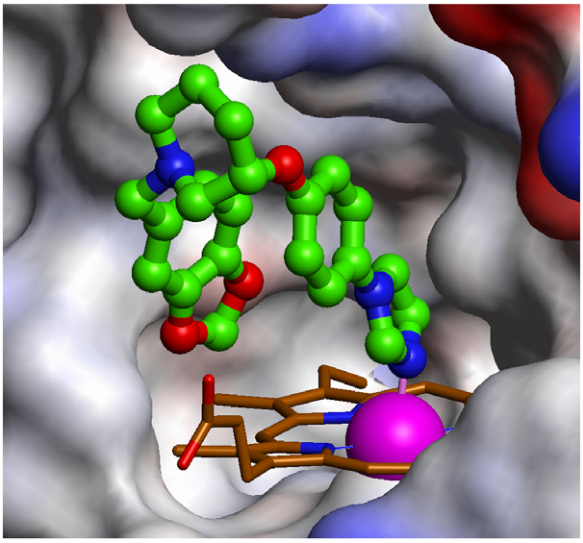


Figure 4. The crystal structure of inhibitor **12S**-murine iNOS Δ114 monomer complex (PDB entry 2ORT).

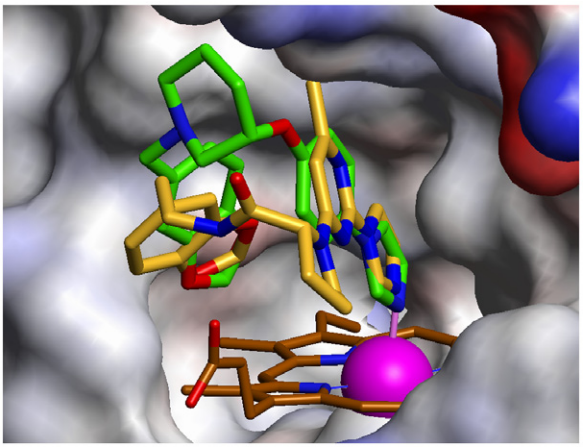


Figure 5. Comparison of crystal structure of inhibitor **12S** (green, PDB entry 2ORT)-murine iNOS monomer complex with inhibitor **1** (gold, PDB entry 2ORO).

In summary, a new series of 1-(1,3-Benzodioxol-5-ylmethyl)-3-[4-(1*H*-Imidazol-1-yl)phenoxy]-piperi-dine analogs have been discovered as inhibitors of NO formation using structure-based design. Compound **12S** potently inhibits iNOS activity in a whole cell assay and demonstrates high iNOS selectivity versus eNOS and nNOS. It is hypothesized that this compound prevents iNOS monomer from dimerizing in a similar fashion to compound **1**.

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