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The rational design of inhibitors of nitric oxide formation by inducible nitric oxide synthase

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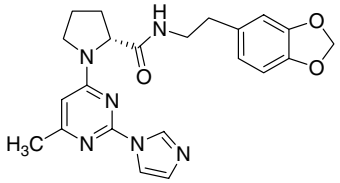
Abstract—A series of compounds was rationally designed as inhibitors of dimer formation of the inducible isoform of nitric oxide synthase, and subsequent nitric oxide production. The conformation of two fragments obtained from a crystal structure was utilized to design a tether connecting those same two fragments. The resulting compounds were potent dimerization inhibitors that bound to the enzyme in a similar conformation as the fragments.

Inducible nitric oxide synthase (iNOS) has been implicated in a variety of immunological diseases such as multiple sclerosis and rheumatoid arthritis. Two constitutive nitric oxide synthase (NOS) isoforms have been identified, endothelial and neuronal NOS (eNOS and bNOS, respectively) which are important for normal function. The search for a selective inhibitor of iNOS has been a priority within the industry and the work addressing a specific enzyme inhibitor has been difficult.[1] We and others have described work, leading to inhibitors of nitric oxide (NO) production, that does not lead directly through enzyme inhibition, but results by not allowing the enzyme to adopt the active dimeric form.[2,3]



1

In our previous report, we described the optimization of a series of imidazolepyrimidines and the discovery of **1**, with an IC_{50} of 0.2 nM in a whole cell assay.[4] The crystal structure of this inhibitor and the murine iNOS monomeric oxygenase domain (iNOS Δ114) in a complex was discussed. The imidazole binds directly with the iron and the compound loops back to position the benzodioxolane moiety over the porphyrin. In fact, the soaking

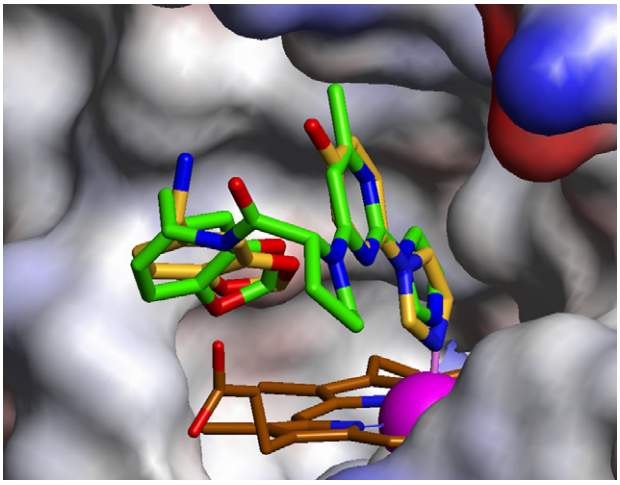


Figure 1. Crystal structure of 4-(imidazol-1-yl)phenol and piperonylamine overlapping (gold carbon atoms, PDB entry 2ORQ) with **1** and bound to murine iNOSΔ114 (green carbon atoms, PDB entry 2ORO).

Keywords: Dimerization inhibitors; iNOS.
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of the two key fragments in the form of 4-(imidazole-1-yl)-phenol and piperonylamine gave a very nearly identical orientation (Fig. 1). A superposition of the two structures shows that the two fragments overlap with **1**, binding in the murine iNOS monomer active site. The arylimidazole moieties overlap nearly completely, while the benzodioxolanes occupy the same space, but are slightly out of plane from one another. The adopted conformation of these two fragments within the enzyme suggested some alternative structures that would interact in a similar manner and potentially disrupt the protein–protein interaction critical for the dimerization process and subsequent activation of the enzyme (Fig. 2). As we wanted the two fragments to be presented to the enzyme in a U-shape, our initial attempts focused on a central template we had used in the past to present functionality in a very similar fashion, 2,6-disubstituted heterocycles.[5]

The compounds with both aromatic groups attached through a heteroatom were prepared by standard methods (Scheme 1).[6] Sequential reaction of the appropriately substituted phenols with the appropriately substituted difluoropyridine or dichloropyrimidine gave the diphenoxyheterocycle. Addition to the pyrimidines typically gave mixtures that were sometimes difficult to separate.

The pyrimidines with one of the aromatic groups attached through a methylene were prepared through the corresponding pyrimidine (Scheme 2). The nitrile of 3,4-(methylenedioxy)phenylacetonitrile was converted to the amidine. Reaction of the amidine with diethyl malonate gave the dihydroxypyrimidine, which was reacted with phosphorus oxychloride to give the dichloropyrimidine. Sequential displacement of the chlorides gave the desired compounds.

Compounds were assayed for inhibition of iNOS in a whole cell assay using human A172 cells induced with cytokines, which has been described previously.[4,7] NO formation was monitored spectrophotometrically with the Griess reagent. The A172 data in the tables refer to the IC_{50} determination as an average of at least two separate experiments.

The initially prepared compounds showed good activity (Table 1). The unsubstituted pyridine, **2a**, had an IC_{50} of 78 nM. Additional substitution of the pyridine, as in **2b**, was detrimental. Pyrimidines were also investigated as the central core and as long as one of the key substituents was straddled by a nitrogen (**2c**–**e**), similar potency

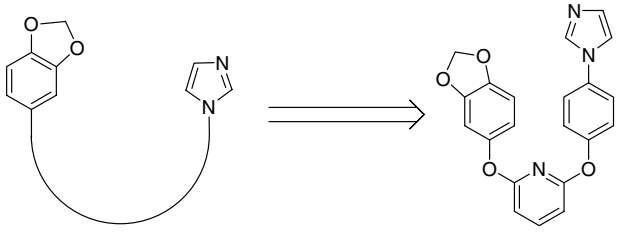
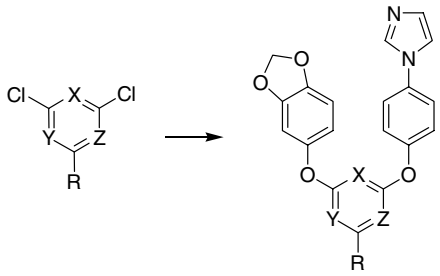
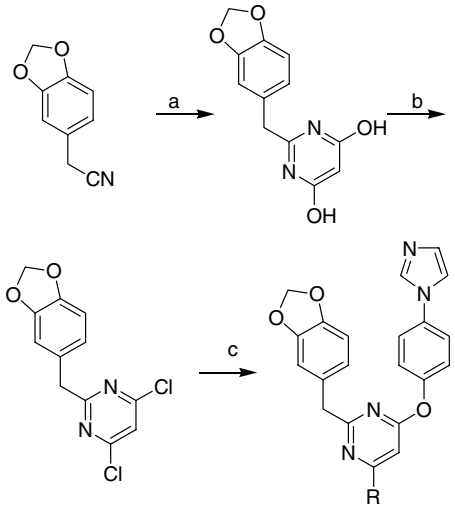


Figure 2. Initial compound design hypothesis.

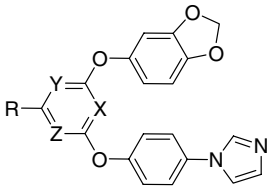


Scheme 1. Reagents and conditions: (i) Sesamol, Cs_2CO_3, DMSO, 50 °C, 18 h; (ii) 4-(imidazole-1-yl)phenol, Cs_2CO_3, DMSO, 90 °C, 18 h.



Scheme 2. Reagents and conditions: (a) (i)HCl, EtOH, 0 °C; (ii)NH_3, EtOH; (iii)diethyl malonate, sodium methoxide, EtOH, reflux, 5 h; (b) POCl3, *N*,*N*-diethylaniline, 45 °C, 3 h; (c) (i)4-(imidazole-1-yl)phenol, K_2CO_3, DMSO; (ii)amine, DMSO, 40 °C, 24 h.

Table 1. Inhibition of NO production in A172 cells[7]



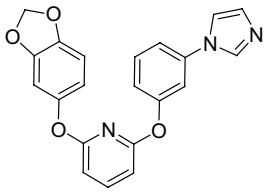
2

Compound	X	Y	Z	R	A172 (nM) IC_{50}
2a	N	CH	CH	H	78
2b	N	CF	CF	CH_3	450
2c	N	CH	N	H	55
2d	N	CH	N	CH_3	90
2e	N	N	CH	H	74
2f	CH	N	N	H	>10,000
2g	N	CCH_3	N	H	130

was realized. The single 4,6-disubstituted compound, **2f**, was inactive. Placement of a hydrogen between the two aromatic moieties may make the U-shaped conformation less favorable. Unlike the pyridine, additional

substitution of the pyrimidine gave a compound with similar activity (**2d**).

In our earlier work on analogously substituted pyridines for Factor Xa,[5] we had found that the analogs that presented the binding substituent in the para position, as is the imidazole, tended to be suboptimal from a potency perspective, presumably due to an inability to best place a substituent. In contrast, the 3-substituted compounds on rotation could adopt a wider range of conformations and were more potent. Preparation of a compound in this case, **3**, showed this not to carry over in this example, as the compound did not attain an IC_{50} at 10 μM.



3

When the structures of the fragments are overlapped with **2c**, the overlap of the benzodioxolane moiety is not as good as that of the previously reported compound **1** (Fig. 3). This is consistent with the experimental results, as the activity difference between **2c** and **1** is approximately two orders of magnitude. The overall structure is similar with the imidazole binding to the iron and the benzodioxolane positioned over the porphyrin. Comparing the structure of **2c** to the two independent fragments, the imidazole overlaps well, but the planes defined by the phenyl and the imidazole rings are further from planarity (6° vs 13° for the dihedral angle between the imidazole and phenyl rings). The benzodioxolane is in the same area over the porphyrin, but the conformation is different. Whereas the planes defined by the benzodioxolane and phenyl of the two fragments are closer to perpendicular (the angle between the plane normals is 65.1°), in **2c** they are closer to parallel (35.2°).

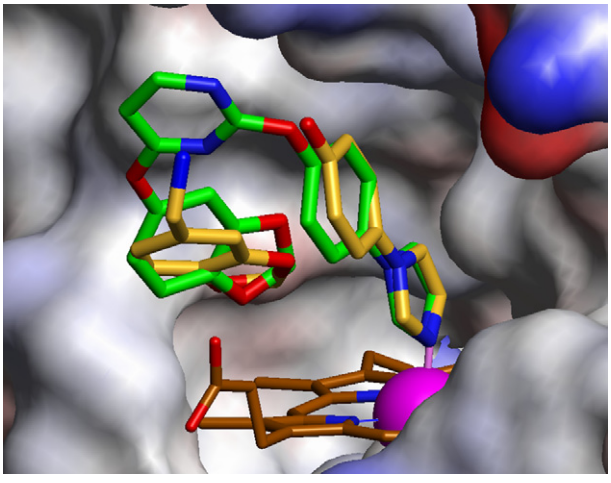


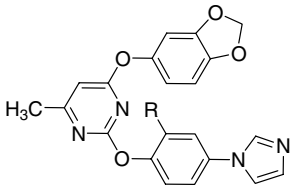
Figure 3. Crystal structure of 4-(imidazol-1-yl)phenol and piperonylamine (PDB entry 2ORQ) overlapping with **2c** bound to murine iNOSΔ114 (PDB entry 2ORR).

These two conformational differences may contribute to the decrease in activity. Additional differences, substitution on the pyrimidine and position of the pyrrolidine, may also contribute to the increase in activity of compound **1**.

Taking into account the structural information and the SAR of **1**, we tried to effect the dihedral angle of the phenyl and imidazole ring by introducing substitution on the phenyl (Table 2). Addition of a small substituent, such as fluoride (**4a**), gave a compound with similar activity, and a larger substituent, trifluoromethyl (**4c**), gave slightly less activity than the hydrogen substituted compound, **2d**. The intermediate chloride (**4b**) gave the most active compound in the series.

To circumvent the chemical selectivity problem associated with the pyrimidine synthesis, an alternate set of compounds was prepared, in which an aromatic moiety was attached to the central template by a carbon (Table 3). In addition, water-solubilizing groups could be added in the 6-position of the pyrimidine to modulate lipophilicity as the second chloride could be displaced and the crystal structure did not indicate any detrimental steric interactions. Unfortunately, significant activity was lost with chloro substituted analog, **5a**. This loss of activity was surprising in light of the activity of **2e** and **2g**. The addition of nitrogen-containing substituents brought back some of the activity.

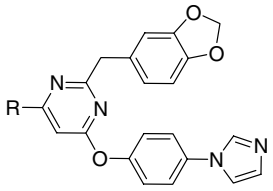
Table 2. Inhibition of NO production in A172 cells[7]



4

Compound	R	A172 (nM) IC_{50}
2d	H	90
4a	F	92
4b	Cl	19
4c	CF_3	220

Table 3. Inhibition of NO production in A172 cells[7]



5

Compound	R	A172 (nM) IC_{50}
5a	Cl	>10,000
5b	Morpholine	830
5c	$N(CH_3)(CH_2)_3N(CH_3)_2$	220

Although we assumed these compounds were acting as dimerization inhibitors, we had no data to differentiate them from direct enzyme inhibitors. Compound **2c** was assayed in standard human enzyme assays[8] and found to have very low activity (>70 μM) against the inducible isoform, certainly not sufficient to explain the activity in the whole cell assay. No activity against either bNOS or eNOS was observed at 100 μM in the corresponding assays.

In summary, we have identified compounds that potently inhibit nitric oxide formation by cytokine induced A172 cells. The compounds were designed by first obtaining a crystal structure of the key fragments bound in the enzyme. A tether was designed that could induce a similar conformation of the two substituents. Although the compounds could be further modified, an alternate tether was identified that had significant advantages and is reported in the subsequent communication.[9]

Acknowledgment

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7. Assay:[4] A172 Cells were harvested and plated. Eighteen to 24 h later, 222 U/ml of human interferon-gamma, 22 ng/ml of human tumor necrosis factor-alpha, 2.2 ng/mL of human interleukin 1-β, and the appropriate concentration of the compound were added. After incubation for 18–24 h, an aliquot of the culture medium was removed and tested for nitrite concentration with Griess reagent. IC_{50} values were calculated from a log-logit analysis of the data.
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