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# Nitric oxide synthase and structure-based inhibitor design

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ABSTRACT

Once it was discovered that the enzyme nitric oxide synthase (NOS) is responsible for the biosynthesis of NO, NOS became a drug target. Particularly important is the over production of NO by neuronal NOS (nNOS) in various neurodegenerative disorders. After the various NOS isoforms were identified, inhibitor development proceeded rapidly. It soon became evident, however, that isoform selectivity presents a major challenge. All 3 human NOS isoforms, nNOS, eNOS (endothelial NOS), and iNOS (inducible NOS) have nearly identical active site structures thus making selective inhibitor design especially difficult. Of particular importance is the avoidance of inhibiting eNOS owing to its vital role in the cardiovascular system. This review summarizes some of the history of NOS inhibitor development and more recent advances in developing isoform selective inhibitors using primarily structure-based approaches.



## 1. Introduction

Not too long after NOS was first isolated [1], it was recognized that elevated levels of NO might be associated with the well known "hot dog headache" and "Chinese restaurant syndromes" [2]. The first is due to high levels of nitrate and the second to monosodium glutamate. The connection between the two is NO since nitrates can be reduced to NO while glutamate is a neurotransmitter that can stimulate NO production. Thus the elevation of either one or both results in the various headache syndromes associated with foods high in nitrates or glutamate. Elevated levels of NO also is associated with migraine headaches [3] and it was postulated quite early on [2] that amino acids including glutamate play a role in Alzheimer's disease by elevating NO levels. The obvious culprit should be neuronal NOS (nNOS). Studies with NOS mouse knockouts indicated that nNOS does, indeed, play an important role in promoting tissue damage after experimentally induced stroke [4]. On the other hand, eNOS plays a protective role. Thus targeting NO over production in neurodegenerative diseases requires isoform-selective drugs that preferentially inhibit nNOS over eNOS. Although sequences clearly showed strong conservation among the three mammalian NOS isoforms, it was not until the crystal structures were solved that it became apparent that isoform selective drug design would be a challenging problem.

## 2. Structural biology

Structure-based inhibitor development began in earnest when the crystal structures of the heme oxygenase domain for all 3 mammalian isoforms were solved [5–9]. This was followed by FAD/FMN reductase C-terminal half of NOS [10] and the FMN module complexed with calmodulin [11]. The crystal structure of holo-NOS has been illusive given the modular architecture of NOS and the large motions required for activity. It now is generally accepted that NOS must undergo a large rearrangement of modular units in order for the FMN module to properly dock to the heme domain for electron transfer (Fig. 1). Knowing the crystal structures of the various NOS modules together with cryoEM [12–14], hydrogen-deuterium exchange [15], molecular dynamics [16,17], and a wealth of mutagenesis data has provided a working model of holo-NOS and the role calmodulin plays in NOS activation [18]. The crystal structure of the nNOS reductase domain is very similar to P450 reductase and in both structures the FMN and FAD are in direct contact. Therefore, in order for the FMN domain to dock to the heme domain as shown in Fig. 1, the FMN and FAD modules must separate followed by a large reorientation of the FMN module relative to the FAD module. Current modeling efforts do not account for the location of the FAD domain and is presumed to be orientationally disordered and does not participate in docking to the heme domain.

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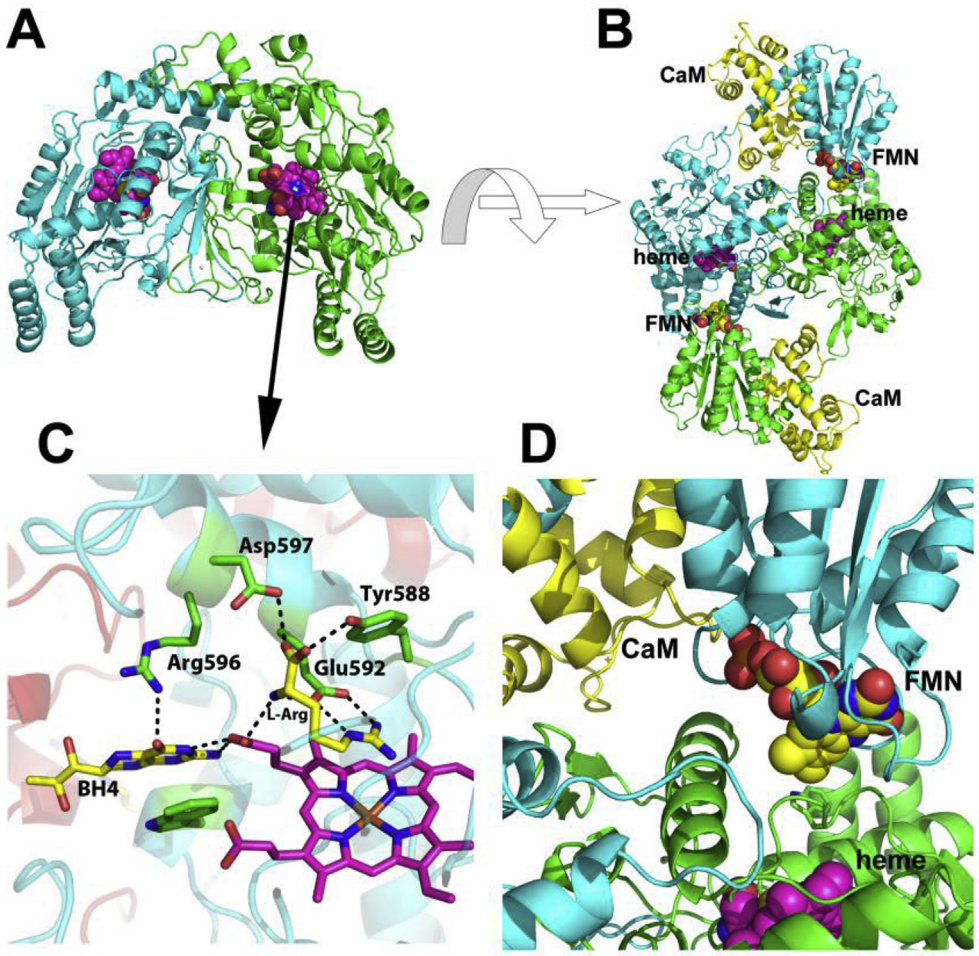
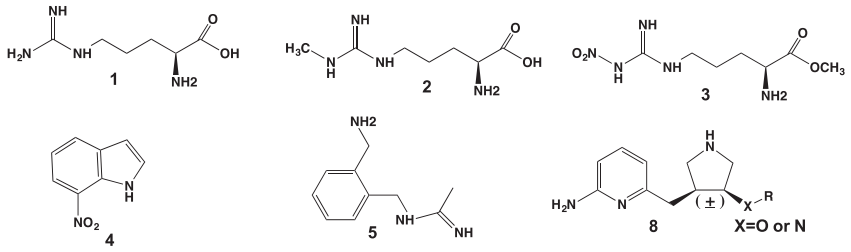



**Fig. 1.** Mammalian NOS structure. A) The rat nNOS heme domain dimer (PDB code 1OM4). The magenta sphere model is the heme. B) Docking model of the complex formed between the iNOS heme and FMN domains [17]. The FMN domain of the molecule A (cyan) docks to the heme domain of molecule B (green) and vise versa. Calmodulin is yellow. C) The NOS active site showing the interactions between the protein and substrate, L-Arg, and the cofactor, $BH_4$. D) Close-up view of the docking interactions between the FMN and heme modules. In this model calmodulin directly interacts with the heme domain which helps to stabilize the FMN-heme interaction, required for efficient electron transfer.

### 2.1. Early NOS inhibitors




Prior to the crystal structures it had been established that compounds based on arginine (**1**) inhibit NOS and exhibit neuroprotective properties. For example, $N^G$-mono-methyl-L-arginine (**2** L-NMMA) and $N^G$-nitro-L-arginine methyl ester (**3** L-NAME) exhibit neuroprotection in such conditions as cerebral stroke and Parkinson's disease [19].

However, these inhibitors also resulted in hypertensive effects [20] most likely due to inhibition of eNOS which is not surprising given that neither **2** nor **3** are isoform-selective *in vitro*. One of earliest compounds found to exhibit selectivity is 7-nitroindazole (7-NI **4**). Initial crystallographic studies showed that 7-NI binds in the eNOS active site and changes the orientation of the active site Glu while 3-bromo-7-NI can bind in both the active and pterin sites [21]. Additional structural studies with both eNOS and iNOS with several other nitroindazoles found similar changes [22]. Despite the claim that 7-NI is nNOS selective, *in vitro* studies showed that 7-NI binding to eNOS and nNOS is about the same [23]. Even so, 7-NI has no effect on eNOS activity in intact blood vessels but does inhibit nNOS in intact cerebellar slices [23] which very likely means that 7-