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Publication Date
2017-02-01

DOI
10.1016/j.niox.2016.11.004

HHS Public Access

Author manuscript

Nitric Oxide. Author manuscript; available in PMC 2018 February 28.

Published in final edited form as:

Nitric Oxide. 2017 February 28; 63: 68–77. doi:10.1016/j.niox.2016.11.004.

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.

Keywords

Nitric Oxide Synthase; Structure Based Drug Design; Isoform Selectivity; Neurodegeneration; Melanoma

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

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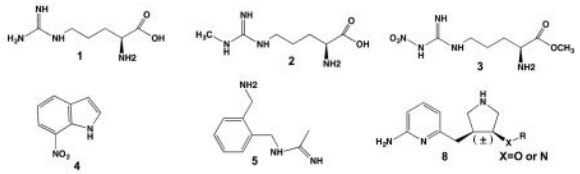
other hand, eNOS plays a protective role. Thus targeting NO over production in neurodegenerative diseases requires isoform-selective drugs that preferentially inhibit nNOS over eNOS. Although sequences clearly showed strong conservation among the three mammalian NOS isoforms, it was not until the crystal structures were solved that it became apparent that isoform selective drug design would be a challenging problem.

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.

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-NI cannot penetrate into endothelial cells to inhibit eNOS. 7-NI also was found not to effect the cardiovascular system [24]. Later studies, however, found that 7-NI does not effect blood pressure in anesthetized animals but lowers blood pressure in animals that do not receive any anesthesia [25] suggesting a complex interplay between experimental variables. Overall it would appear that claims of 7-NI being selective for nNOS were over stated.

A second inhibitor reported to be selective for iNOS is 1400W (**5**) [26]. 1400W is an irreversible inhibitor (inactivator) of iNOS but not eNOS and nNOS. The initial K_i for 1400W binding to iNOS and nNOS are similar and the crystal structures of 1400W bound to all three isoforms are nearly identical [27,28] indicating that the selectivity is not due to a greater affinity for iNOS. The basis for selectivity is thus the ability of iNOS, but not eNOS or nNOS, to activate 1400W resulting in a reactive intermediate that covalently modifies and inactivates iNOS. This is similar to what happens when iNOS is treated with the irreversible inhibitor N^5-(1-iminoethyl-L-ornithine that results in heme destruction [29]. Why iNOS is more susceptible to mechanism based covalent inactivation is probably associated with the much faster rate of NO formation iNOS compared to the other two isoforms [30] thereby increasing the chances of inhibitor activation at the heme center resulting in local covalent modification.

Discovery of nNOS Selective Inhibitors: Pre Crystal Structures

Early on L-nitroarginine was reported to be about 300-fold more selective for nNOS over iNOS but not eNOS [31]. In fact, a majority of the early inhibitors behaved similarly and exhibited little difference in binding between nNOS and eNOS. This precluded the therapeutic application of such inhibitors owing to the deleterious effects on the cardiovascular system by inhibiting eNOS. This lack of selectivity was not surprising since even without crystal structures, it was clear from sequence alignments that the active site of all 3 isoforms are nearly identical. However, Nature can tolerate greater sequence variability near the entrance of the active site pocket so if an inhibitor could be prepared that anchors one end in the active site with the tail end extending out of the active site, it might be possible to achieve some level of selectivity. Given that the substrate is L-arginine, straightforward peptide synthesis to give dipeptides might give an inhibitor where the L-arginine-like half of the dipeptide is positioned well within the active site while the second amino acid integrates regions near the surface where sequence variations are better tolerated. This logic proved correct in 1997 when some L-nitroarginine dipeptides were found to be as much as 1800-fold more selective for nNOS over iNOS [32]. Similar studies with thiocitrulline dipeptides resulted in ≈ 70-fold selectivity of nNOS over eNOS [33]. These successes resulted in a much larger study which led to the discovery of a dipeptide that is ≈ 1500-fold (**6**, Fig. 2) more selective for nNOS over eNOS [34]. Shortly after this breakthrough, the crystal structures became available thereby initiating structure based inhibitor design.

The Structural Basis for Isoform Selectivity

With the crystal structures and highly selective dipeptide inhibitors in hand, it was fairly straightforward to establish the structural basis for isoform selectivity [35]. The structure of **6** bound to bovine eNOS and rat nNOS showed (Fig. 2) that **6** adopts quite different conformations in the two isoforms. In nNOS, the inhibitor "curls" which enables the inhibitor α-amino group to directly H-bond with the conserved active site Glu. In eNOS the inhibitor adopts an extended conformation resulting in a water molecule bridging between the active site Glu and the inhibitor α-amino group. The most obvious difference in the active site that might be responsible for both the difference in conformation and affinity is that where nNOS has Asp597 eNOS has Asn368. As a result, nNOS has two negatively charged side chains, Asp597 and Glu592, that can electrostatically stabilize the inhibitor α-amino group. To optimize these interactions the inhibitor must "curl" thereby placing the α-amino group in an optimal position between Asp597 and Glu592. In sharp contrast, Asn368 in eNOS bears no negative charge. This hypothesis was tested by making the Asp597Asn mutant in nNOS and Asn368Asp mutant in eNOS [35]. For one group of these dipeptide inhibitors where crystal structures of both wild type and mutant complexes could be solved, the eNOS Asn368Asp mutant dropped K_i from 110 μM to 21 μM and the inhibitor bound with curled conformation as in the wild type nNOS while in the nNOS Asp597Asn mutant K_i increased from 0.10 μM to 5.1 μM and the inhibitor bound with extended conformation as in the wild type eNOS. It also was noted that the tail end of the inhibitor extended out toward Val106 in eNOS and Met366 in nNOS leading to the analysis of double mutants. In the eNOS Asn368Asp/Val106Met double mutant K_i dropped another 5-fold [36]. The rationale for this difference is that the larger Met side chain forms better non-bonded interactions with the tail end of the inhibitor. These results showed that a mere 2 amino acid difference comes within a factor of 10 of explaining the over 1,000-fold selectivity of these dipeptides for nNOS over eNOS.

Second Generation nNOS Inhibitors

Despite the selectivity of the dipeptide inhibitors, the extensive positive charge and potential lability of peptide bonds were viewed as limiting bioavailability. As a result, lessons learned from the dipeptide inhibitor effort were applied to the design and synthesis of the next generation of inhibitors with improved bioavailability properties. A good starting place was to substitute the guanidino end of L-Arg like inhibitors with an aminopyridine especially since aminopyridines had been shown early on to inhibit NOS [37,38]. Moreover, the near neutral pKa of aminopyridines decreases total charge and thus might improve bioavailability.

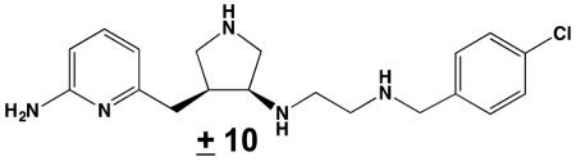
One of the first structural studies of aminopyridines bound to nNOS [39] showed that compounds like **7** (Fig. 3) were positioned with the aminopyridine over the heme of iNOS near the active site Glu377 as expected. In the inhibitory assays **7** exhibited about 270-fold selectivity for iNOS over eNOS while there was little difference between eNOS and nNOS. Crystal structures led to the idea of anchored plasticity [39]. One end of the inhibitor is "anchored" over the heme near the active site Glu and will be the same for all NOS isoforms given the strong structural conservation. However, the other end of the inhibitor will explore the more "plastic" region of the active site near the surface where greater flexibility in one

isoform will lead to improved binding. As sown in Fig. 3, residues, Arg266 and Gln263 in iNOS, are able to adopt alternate conformations thus creating a new binding pocket for the tail end of the inhibitor.

At about the same time the first compounds to take advantage of what was learned from the dipeptide inhibition work and the Asp/Asn difference between nNOS and eNOS were compounds related to **8** [40]. This also was one of the first attempts to use computational methods to help in the design of selective inhibitors giving rise to a novel “fragment hopping” methodology [40]. The NOS active site is divided into pharmacophores and then various fragments docked into these sites while taking into account toxicity and metabolic stability. The aminopyridine end of the inhibitor should interact with the active site Glu while the pyrrolidine N atom should interact with Asp597 in nNOS but much more weakly with Asn368 in eNOS. The crystal structures showed that compounds like **8** bound to rat nNOS very close to what was predicted (PDB 3B3M and 3B3N) [41].

Further studies explored variations of **8** with different R groups ultimately leading to the discovery of how important chirality is to isoform selectivity. The pyrrolidine ring has two chiral centers giving rise to 4 possible diastereomers, (R,R), (R,S), (S,R), and (S,S). (3′R, 4′R)-**9** (Fig. 4) was found to exhibit 3,800-fold selectivity for rat nNOS over bovine eNOS while the (3′S,4′S)-**9** exhibited only 505-fold selectivity [42]. Moreover, (3′R,4′R)-**9** binds about 10-fold more tightly to nNOS than (3′S,4′S)-**9**. Crystal structures revealed that (3′R, 4′R)-**9** and (3′S,4′S)-**9** adopt totally different binding modes. (3′S,4′S)-**9** binds as expected with the aminopyridine in the active site interacting with the active site Glu in both nNOS and eNOS [42]. However, in both eNOS and nNOS (3′R,4′R)-**9** flips 180° which places the fluorophenyl group in the active site while the aminopyridine H-bonds with the heme propionate. In order for this to happen Tyr706 must adopt a new rotamer (Fig. 4). Although (3′R,4′R)-**9** binds in the same flipped orientation in both eNOS and nNOS, the selectivity over eNOS is nearly 4,000-fold making this one of the most selective inhibitors discovered. The nNOS Asp597Asn mutant increased K_i from 5.3 nM to 29 nM which does account for only a small part of the nearly 4,000-fold selectivity. One additional contributing factor observed using the Tyr to Ala mutants of both nNOS and eNOS is that Tyr706 forms more extensive non-bonded interactions with the aminopyridine in nNOS than in eNOS but even here the full level of selectivity cannot be rationalized [42]. From the many structures that have been solved and correlated with K_i measurements, it has become clear that a full understanding of selectivity becomes more difficult when significant structural changes are involved. What cannot be observed in static crystal structures is relative active site flexibility. For example, Tyr706 must swing out of the way in order for (3′R,4′R) to bind which further means that Tyr706 must spend at least part of the time in the “out” orientation in order to make the heme propionate available for inhibitor binding. The relative flexibility of Tyr706 in nNOS and Tyr477 in eNOS then could contribute significantly to selectivity.

Animal models

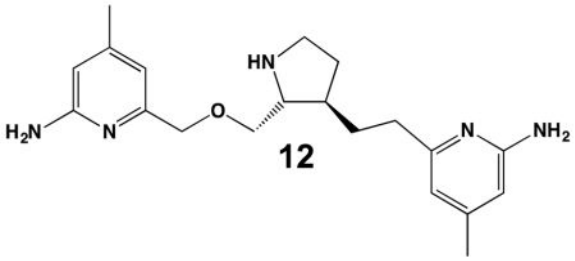


The aminopyridine **10** has been extensively tested in an *in vivo* animal model for cerebral palsy. In this model rabbit fetuses are subjected to uterine ischemia at 22–27 weeks of gestation [43]. This results in brain damage owing to increased reactive oxidizing species including NO and newborn kits exhibit cerebral palsy symptoms. Dams received saline or **10** *via* the descending aorta 30–40 min prior to uterine ischemia [44,45]. Also included in these studies was 7-NI (**4**). Treatment with **10** resulted in 62% normal, 7NI 31%, and the saline control 20%. In addition, **10** treated animals performed better in various behavioral tests and **10** also was far superior than 7NI in decreasing NOS activity in fetal brains [45]. Very importantly, **10** had no effect on the dam's blood pressure or heart rate while 7NI did. This illustrates that, unlike what was previously believed, 7NI is not an nNOS-selective inhibitor *in vivo* while **10**, developed using structure-based approaches, is very effective. An aminopyridine closely related to **10** also proved quite effective in neural protection in a sheep model. Umbilical occlusion in pregnant sheep results in fetal brain seizures and neuronal damage. The pre-administration of the aminopyridine significantly decreases neuronal loss owing to asphyxia [46].

Double Headed Symmetrical Inhibitors

Despite the impressive selectivity of **9** and related compounds, these compounds require multistep syntheses and the high charge and H-bonding groups continues to present bioavailabilty problems. These issues were partly addressed by developing simplified inhibitors that takes advantage of what was learned from the (3′R,4′R)-**9** work. Since (3′R, 4′R)-**9** showed that aminopyridines can interact with the heme propionates, a symmetrical double headed inhibitor with an aminopyridine at each end was designed [47]. Such double headed inhibitors should have the ability to interact both with the active site Glu and heme propionates (Fig. 5). Compound **11** has no chiral center, the pKa of all groups is near neutrality, and **11** showed better cell permeability than the parent aminopyridines [47]. The rat nNOS-**11** crystal structure revealed an unexpected surprise [48]. Two molecules of **11** bind. The first binds exactly as predicted with one aminopyridine in the active site and the other interacts with a heme propionate. With the second molecule one aminopyridine displaces the pterin cofactor and interacts with the second heme propionate. This places the bridging pyridine in position to provide one of the ligands for a new tetrahedral Zn^{2+} binding site. The remaining ligands are Asp600, His692 from molecule B of the nNOS dimer, and a chloride ion [48]. In eNOS only the first molecule in the active site binds. Some of the obvious mutants were generated to either prevent or promote binding of the second molecule in eNOS and nNOS, respectively, but to no avail. The only explanation provided thus far is that the eNOS dimer interface may be more rigid than in nNOS which prevents the required adjustments needed to form the Zn^{2+} coordination sphere [48]. Although **11**

binds well to nNOS with a K_i = 25 nM, it exhibits only a 107-fold selectivity over eNOS [47]. The best that has been achieved so far with double headed symmetric inhibitors is about 200-fold selectivity. Although these double headed symmetric compounds provided interesting insights into the potential for targeting the pterin site, the low selectivity over eNOS tempered enthusiasm for further biological studies. This resulted in compounds like **12** which are double headed but not symmetric. **12** binds well to nNOS, K_i = 9.7 nM and exhibits about 690-fold selectivity over eNOS [49].



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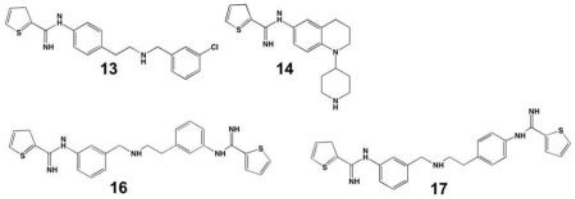
Thiophenecarboximidamides



Thiophenecarboximidamides also have the potential for interacting with the active site Glu similar to the aminopyridines and some have proven to be excellent NOS inhibitors. The structures of **13** complexed to iNOS and nNOS show that the thiophene end of the inhibitor binds in the active where it can interact with the active site Glu [50]. **13** has an IC_{50} = 35 nM for nNOS and exhibited good biological properties since the injection of **13** into rats resulted in high levels in brain [51]. Although the selectivity over eNOS is only about 100-fold and **13** increases mean arterial blood pressure owing to eNOS inhibition, the excellent uptake into the brain focused future studies on this group of NOS inhibitors. Improvement in selectivity resulted in **14** with an IC_{50} = 100 nM for nNOS and about 370-fold selectivity over eNOS [52]. **14** also exhibited good oral availability and functioned well in an *in vivo* pain model [52]. Thus far the most selective thiophenecarboximidamide is **15** (Fig. 6), a double headed compound, with a K_i = 14.7 nM for rat nNOS and 1,334-fold selectivity over bovine eNOS [53]. The crystal structures (Fig. 6) show that one thiophenecarboximidamide is positioned over the heme and H-bonds with the active site Glu similar to the aminopyridines. In nNOS the linker methylamino side group forms a strong H-bond with the heme propionate. The second thiophenecarboximidamine of the inhibitor is well ordered in nNOS. However, in eNOS the electron density is weak making exact positioning of the tail difficult although it is clear that the methylamino cannot directly H-bond with the heme propionate. The better interaction with the heme propionate in nNOS is the most likely reason for the high level of selectivity. To support this H-bond with heme the tail end of the thiophenecarboximidamide in nNOS is able to nestle in a pocket part of which is formed by

Ser602. eNOS has a His373 in place the Ser in nNOS. As a result, the pocket is too small to accommodate the tail thiophenecarboximidamide in eNOS causing disordering.

Although the development of both the aminopyridine and thiophenecarboximidamide inhibitors have been designed with the goal of inhibiting nNOS in neurodegenerative diseases, it recently has been found that nNOS might be a viable target in melanoma. Various melanoma cell lines were found to express high levels of nNOS and the addition of exogenous NO promotes proliferation of melanoma cells [54]. Melanoma cells used in mouse xenograft studies showed that cells with nNOS knocked down generate smaller tumors [54]. These observations encouraged the screening of selective nNOS inhibitors for anti-melanoma properties. The initial screening of aminopyridines showed promising results in the ability to decrease cell invasiveness and NO production in melanoma cells. This was followed by testing a new generation of thiophenecarboximidamides. Compounds **16** and **17** exhibited anti-melanoma activity with EC_{50} values of 1.3 and 1.4 μM, respectively, to be compared with 4.2 μM for cis-platin [55]. **17** exhibits a K_i = 5 nM for nNOS and is about 540-fold more selective over eNOS.

Dimer inhibition

Another approach for inhibiting NOS is to either disrupt the active NOS dimer or prevent NOS dimerization. Knowing that the NOS dimer is stable once formed, such inhibitors should work best by preventing dimer assembly. Given that iNOS is controlled at the transcriptional level, NOS dimerization inhibitors might be expected to work best with iNOS. Indeed, the first generation of such inhibitors were found to prevent iNOS dimerization in cell-based assays [56]. Subsequent studies showed that bulky pyrimidine imidazole inhibitors can promote monomerization of iNOS dimers *in vivo* [57]. Structure-based approaches also were used to design dimerization inhibitors (Fig. 7). The crystal structure of the two end aromatic fragments bound to murine iNOS (2ORQ) were used as a guide to develop a U-shaped linker resulting in **18** [58,59]. Precisely why **18** or any of the other dimer inhibitors prevents dimerization or disrupts the dimer is not clear. The imidazole end of the inhibitor coordinates the heme iron as expected but the tail end of the inhibitor does not extend out of the active site where it might potentially interfere with dimer formation. Instead the tail wraps back into the active site where it interacts with the active site helix that contains the conserved active site Glu377. This region (red helix in Fig. 7) is disordered in the monomeric iNOS-**18** complex. Since the disordered region extends out toward the surface near the dimer interface, **18** may be destabilizing regions of the structure important for the dimer stability [57].

Bacterial NOS

Bacterial NOS (bNOS) has recently emerged as therapeutic target. Certain Gram-positive bacteria have bNOS and two of these are important human pathogens, *B. anthracis*, the causative agent of anthrax, and methicillin resistant *Staph. aureus* (MRSA). Gusarov et al. [60] found that the NO generated by bNOS helps to protect the bacteria from a number of antibiotics. The mechanism appears to involve protection by NO against antibiotic induced oxidative stress although precisely how is not well understood. Studies with ΔNOS strains

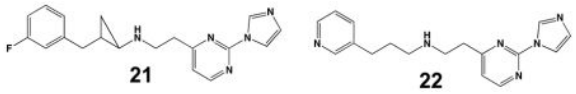
also showed that bNOS is essential for *B. anthracis* virulence [61] and bNOS also plays a role in protecting MRSA from oxidative stress [62]. The generic and non-specific NOS inhibitors N-methyl-L-arginine and N-nitro-L-arginine rendered bacteria more susceptible to oxidative stress [63] which was the first example that bNOS might be a viable drug target. Since a large number of compounds were available as part of the drug discovery program centered on neurodegeneration [64,65], a number of these inhibitors were screened against both *B. subtilis* [66] and MRSA [67] and a select few worked synergistically with antibiotics or H_2O_2 induced oxidative stress to kill bacteria. Compound **19** (Fig. 8) was particular impressive. **19** alone had very little effect on *B. subtilis* growth while the antibiotic, acraflavin, slowed growth only 20% but the antibiotic together with **19** at the same concentrations used for the controls slowed growth by ≈ 80% [66].

Crystal structures provided some clues on how to exploit differences between bNOS and the mammalian NOS isoforms. **19** binds to bNOS and nNOS with similar affinities, K_S (spectral dissociation constant) = 1.1 and 0.4 μM, respectively [66]. However, **19** flips 180° in bNOS relative to nNOS. In both structures one aminopyridine binds over the heme to H-bond with the conserved active site Glu. In nNOS the second tail-end aminopyridine interacts with heme propionate D which can only happen when Tyr706 swings out of the way. In bNOS, however, the tail end aminopyridine interacts with heme propionate A which requires displacement of the BH_4 used in crystallization. One reason for this difference is that bNOS binds BH_4 much more weakly than nNOS, μM range in bNOS [69] and nM range in mammalian NOS [70]. As a result, the inhibitor can more easily displace the BH_4 in bNOS and in order to best optimize binding in the BH_4 site, **19** flips over relative to nNOS. As shown in Fig. 8, bNOS has a much more open BH_4 pocket given that bNOS does not have the Zn^{2+} binding motif along the dimer interface found in the mammalian isoforms. These studies have directed attention to the bNOS BH_4 pocket as a potential druggable target.

Humanizing NOS Inhibitor Design

The *in vitro* inhibitory assays using isolated NOS enzymes coupled with the structural characterization of the NOS-inhibitor complex have efficiently generated various compounds with good potency and isoform selectivity. Given the goal of discovering drugs for human disease, recent efforts have been more focused on whether compounds (1) are potent and selective for both lower animal and human targets, and (2) show better oral bioavailability and membrane permeability.

Recently, the human nNOS and eNOS crystal structures have become available at good resolution [71]. Although rat nNOS and human nNOS share 94 % sequence identity, a detailed comparison indicates that in a peripheral pocket next to the active site Leu337 in rat is replaced by His342 in human [72]. This pocket influences the binding mode of the tail end in bulky inhibitors. Design of nNOS selective inhibitors that are potent for both rat and human is of importance for successful preclinical animal studies that can eventually lead to the final clinical human trials.



Achieving good bioavailability has proven to be an even more challenging task than finding potent and selective inhibitors *in vitro*. As outlined in this review, one approach has been to try different arginine isosteres with better physiochemical properties. Most recently the 2-aminoquinoline group (Fig. 9) was chosen to replace 2-aminopyridine [73,74]. Compounds were first screened with *in vitro* inhibitory assays and optimized *via* structural approaches against both rat and human enzymes. **20** has a K_i = 58 nM for nNOS and exhibits 216-fold selectivity over eNOS [74]. The oral bioavailability of the best candidates were further tested using Caco-2 cell permeability assays [75] to approximate the permeability of the gut epithelium. For nNOS specific inhibitors attention was also paid [74] as to whether compounds showed any off-target binding to other receptors in the central nerve system (CNS) *via* the Psychoactive Drug Screening Program (PDSP) [76]. One of the more recent studies has focused on imidazole inhibitors that coordinate the heme iron [77]. Inhibitor **21** exhibits good Caco-2 cell permeability and is about 360-fold more selective for nNOS over eNOS. While promising, an important consideration is the ability of such inhibitors to also coordinate the heme iron of cytochromes P450. Indeed, while **21** shows promise as a nNOS inhibitor, **21** is only 17-fold more selective for nNOS over CYP3A4, the main drug metabolizing P450 [77]. **22** provides a good compromise. The K_i for **21** binding to nNOS is 0.018μM compared to 0.054μM for **22** yet **22** is 200 fold more selective for nNOS over eNOS and 1296-fold over CYP3A4.

Conclusions

The development of NOS inhibitors has proceeded at a moderate pace owing primarily to bioavailability issues. The NOS active site is polar as it must be in order to bind L-Arg which means inhibitors that mimic L-Arg binding also are highly polar thus limiting bioavailability. This is an especially important problem in targeting nNOS owing to the requirement of penetrating the blood brain barrier. Nevertheless, important lessons have been learned and the fundamental problem of what structural features can be exploited for isoform-selective drug design has been solved. Differences between NOS isoforms in protein dynamics and flexibility also are important but are difficult to address experimentally. However, with the major advances that have been made in computational software and hardware, dynamics of large proteins like NOS now can be explored on biologically relevant time scales. Finally, it is important to explore other uses of the many compounds that have been developed targeting neurodegenerative diseases where bioavailability may not be as large an issue. The recent findings that some nNOS-selective inhibitors slow melanoma invasiveness and work synergistically with oxidative stress to kill Gram positive pathogens bodes well for broadening the use of NOS inhibitors as potential therapeutic agents.

Acknowledgments

Work in our lab was funded by NIH grant GM57353.

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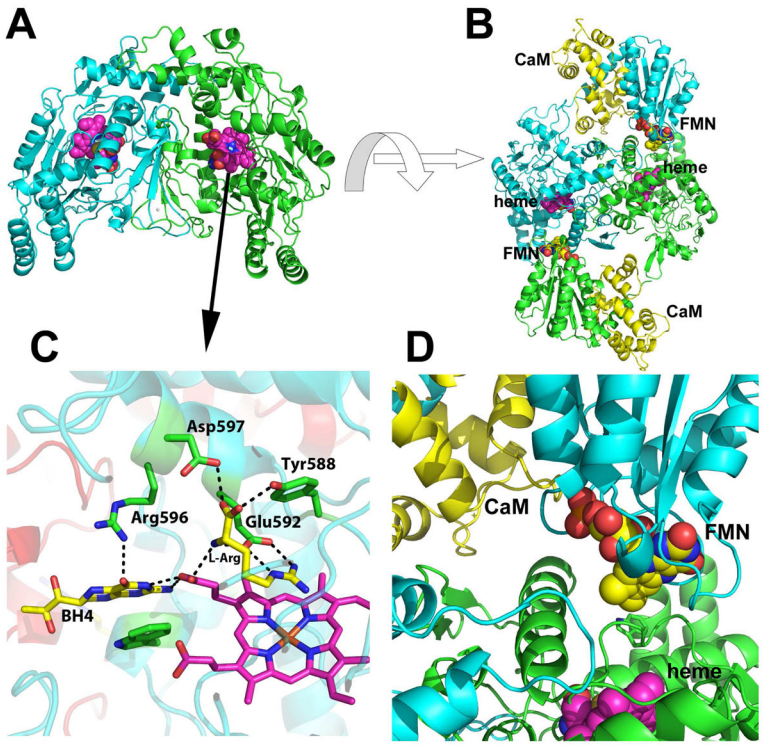

Figure 1.

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.

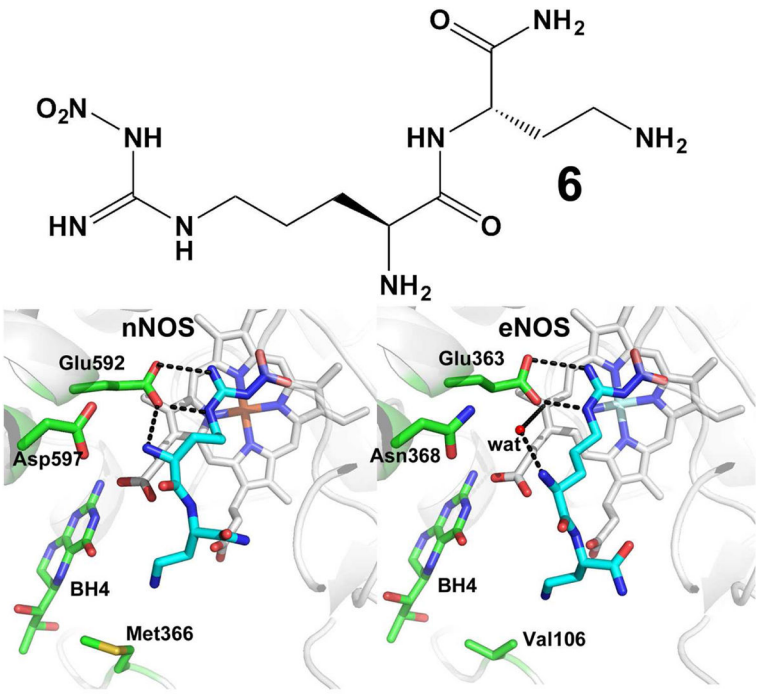

Figure 2.

Crystal structure of dipeptide inhibitor **6** bound to bovine eNOS (1P6L) and rat nNOS (1P6H). Note that in nNOS the inhibitor "curls" which enables the α-amino group to directly interact with Glu592. This places the inhibitor α-amino group in position to stabilize the negative charges on Glu592 and Asp597. In eNOS Asp597 is Asn368 and as a result, there is less electrostatic stabilization in the eNOS-**6** complex when α-amino group is farther away from Glu363.

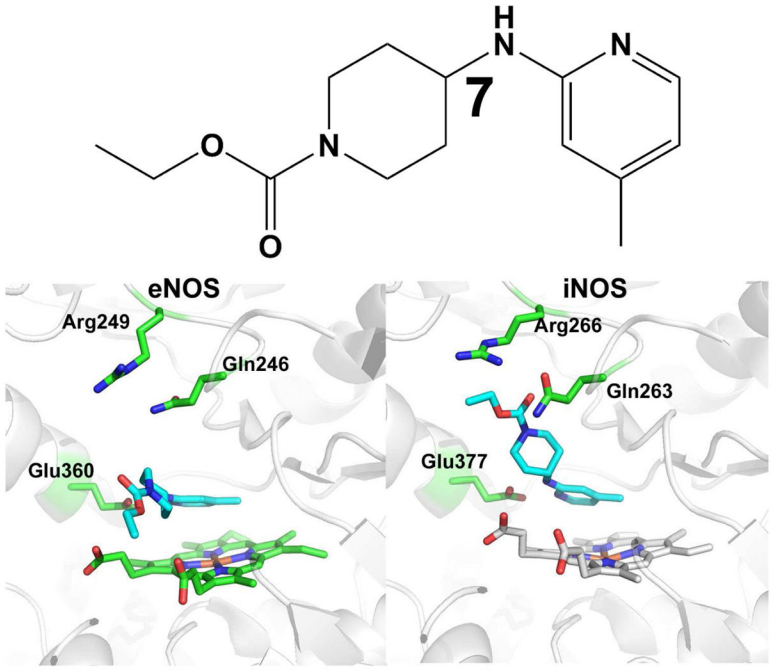

Figure 3.

The structure of **7** complexed to bovine eNOS (3E7S) and human iNOS (3E7G). The inhibitor adopts substantially different conformations in each. This has been attributed to the ability of Gln263 in iNOS to adopt an alternate rotamer conformation while eNOS is more rigid [39]. Such differences in flexibility is one feature that may be exploitable for isoform-selective inhibition.

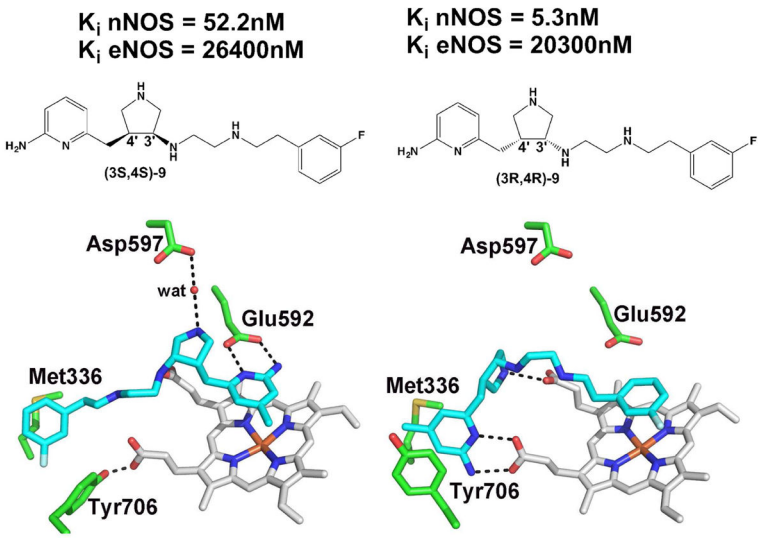

Figure 4.

The importance of chirality in isoform-selective inhibition. (3S,4S)-**9** (3JWS) binds to rat nNOS as expected with the amino pyridine situated over the heme H-bonding with Glu592. However, (3R,4R)-**9** (3JWT) flips 180° enabling the aminopyridine to interact with a heme propionate. This requires Tyr706 to adopt a new rotamer conformation [42].

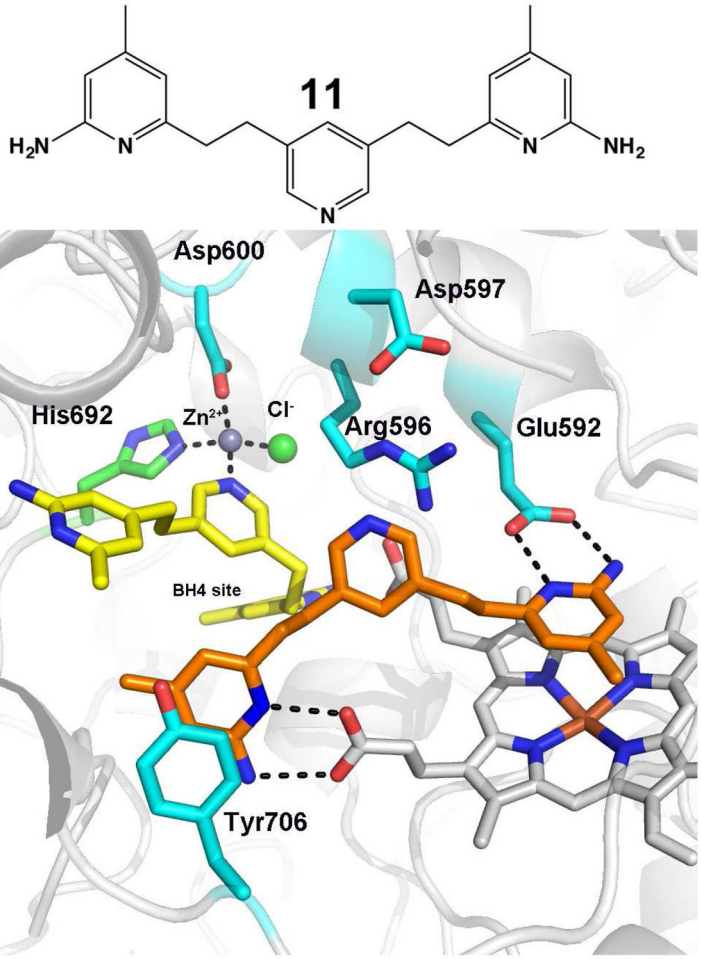

Figure 5.

A simplified symmetric double headed inhibitor **11** (3N5W) [42] that was designed based on what was learned from how **9** binds (Fig. 4). Quite unexpectedly, two inhibitor molecules bind to rat nNOS. One (orange) binds in the active site exactly as predicted but the second (yellow) displaced BH_4 and forms part of a new Zn^{2+} binding site.

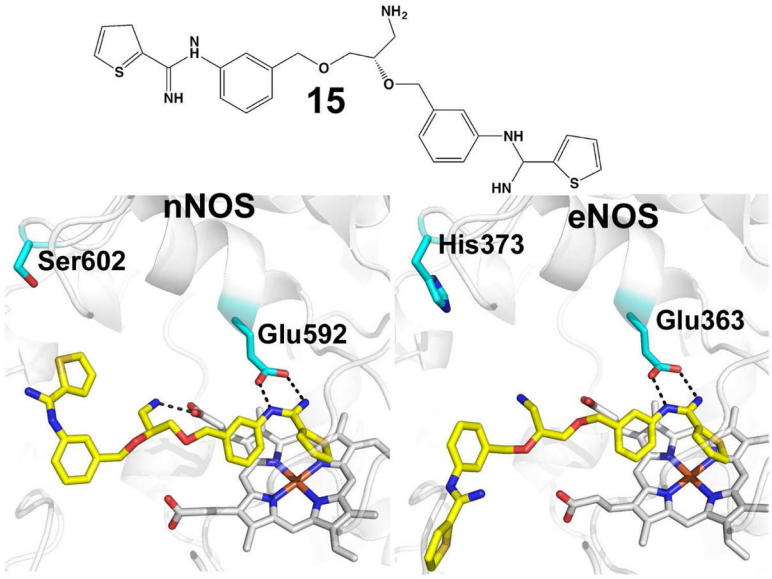

Figure 6.

The tail end of the thiophenecarboximidamide **15** binds differently to bovine eNOS (4UPR) and rat nNOS (4UPN). This may be due to the smaller Ser602 side chain in nNOS which allows the inhibitor to swing up and bind in a small pocket while the larger His373 blocks binding of the tail in eNOS.

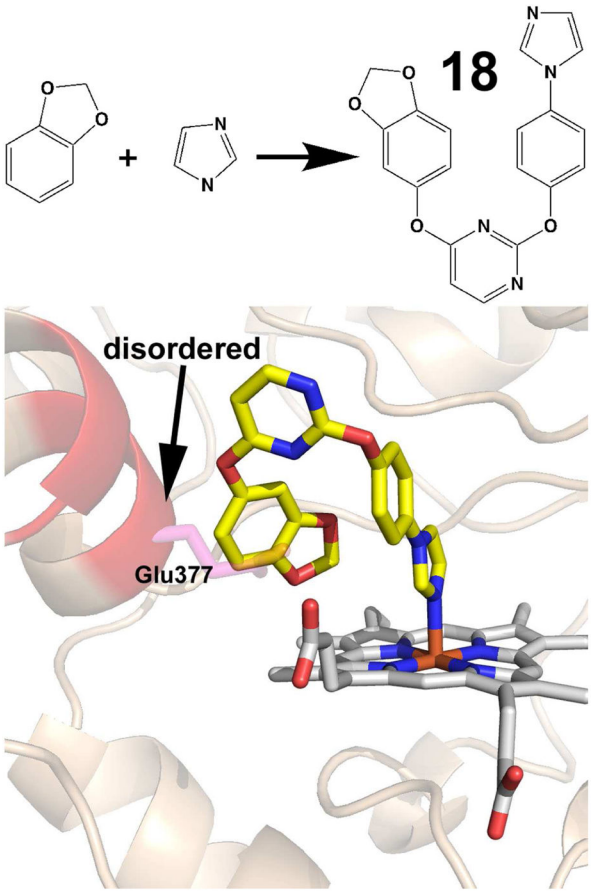

Figure 7.
The binding of **18** to the murine iNOS monomer (2ORR). **18** was found to inhibit iNOS dimerization [58].

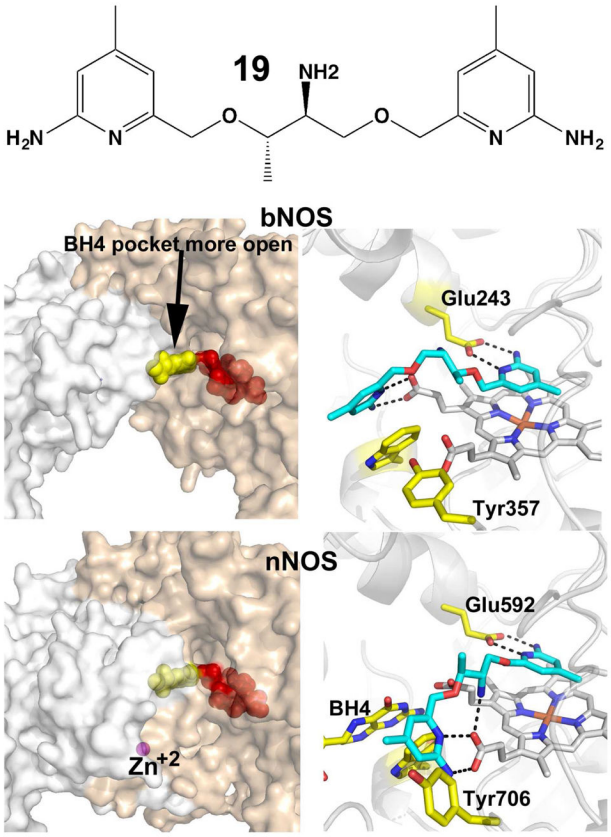

Figure 8.

The binding of **19** to rat nNOS (4K5G) [66] and B. subtilis NOS (bsNOS). In bsNOS **19** flips 180° relative to nNOS. This enables one of the aminopyridines to displace BH_4 in bsNOS. In the mammalian NOS isoforms there is a Zn^{2+} binding motif at the dimer interface that stabilizes the dimer and helps to form the BH_4 binding pocket [68]. This Zn^{2+} motif is missing in bsNOS and as a result, the pterin pocket is more open and binds pterins much more weakly.

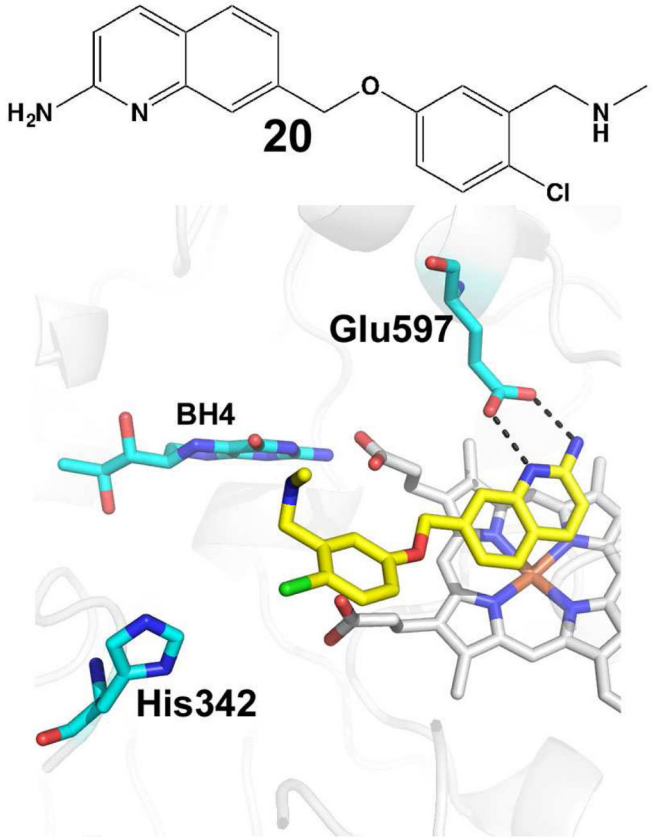

Figure 9.
The binding of aminoquinoline **20** to human nNOS (5ADG).