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Recent Advances Toward Improving the Bioavailability of Neuronal Nitric Oxide Synthase Inhibitors

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Abstract

Overproduction of nitric oxide by neuronal nitric oxide synthase (nNOS) has been highly correlated with numerous neurodegenerative diseases and stroke. Given its role in human diseases, nNOS is an important target for therapy that deserves further attention. During the last decade, a large number of organic scaffolds have been investigated to develop selective nNOS inhibitors, resulting in two principal classes of compounds, 2-aminopyridines and thiophene-2-carboximidamides. The former compounds were investigated in detail by our group, exhibiting great potency and excellent selectivity; however, they suffer from poor bioavailability, which hampers their therapeutic potential. Here we present a review of various strategies adopted by our group to improve the bioavailability of 2-aminopyridine derivatives and describe recent advances in thiophene-2-carboximidamide based nNOS-selective inhibitors, which exhibit promising pharmacological profiles.

Keywords

Bioavailability; Neuronal Nitric Oxide Synthase; Inhibitor; Isoform selectivity; 2-Aminopyridine; Thiophene-2-carboximidamide

INTRODUCTION

Nitric oxide (NO) is one of the most important signaling molecules that regulate many biological processes.[1–3] It is synthesized by nitric oxide synthases (NOSs), which catalyze a reaction of L-arginine, NADPH, and oxygen to give the free radical NO, citrulline, and NADP⁺. To date, three distinct isoforms of NOS have been identified: neuronal NOS (nNOS), endothelial NOS (eNOS), and inducible NOS (iNOS). The three isoforms possess more than 50% homology and differ by distribution in organs and tissues, catalytic properties, and response to different classes of inhibitors.

Neuronal NOS (nNOS) produces NO in nervous tissue in both the central and peripheral nervous system. There is growing evidence demonstrating that NO overproduction by nNOS has been particularly associated with stroke and some chronic neurodegenerative pathologies, supporting its selection as a potential therapeutic target.[4–6] It is noteworthy that inhibition of iNOS and eNOS may cause undesired side effects, and therefore, selectivity of nNOS over the other two isoforms is a key requirement for any potential therapeutic agent.[7–12] Over the past two decades, significant drug discovery efforts have

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been directed toward developing and characterizing selective nNOS inhibitors, both in vitro and in vivo.[9,10,13] Hundreds of nNOS inhibitors from a range of chemical classes, including peptides and small organic molecules, have been disclosed in the literature.[14–19]

In our continuous efforts to develop nNOS selective inhibitors, we discovered a series of highly potent and nNOS-selective small-molecule inhibitors having a 2-aminopyridinomethyl pyrrolidine scaffold.[20] Although some of them showed great potency ($K_i < 10$ nM) and excellent selectivity for nNOS over eNOS (> 2500 -fold) and iNOS (> 700 -fold), they still suffered from some deficiencies, including lack of oral activity, poor membrane permeability, and relative inability to cross the blood-brain barrier (BBB) and access the central nervous system. Therefore, it was essential to enhance their bioavailability in order to facilitate their therapeutic value. In the past few years several strategies were adopted by our group, and dozens of 2-aminopyridinomethyl pyrrolidine based compounds were designed and synthesized to improve membrane permeability without losing potency and/or selectivity.

In addition to the 2-aminopyridinomethyl pyrrolidine derivatives, thiophene-2-carboximidamides represent another important class of selective nNOS inhibitors; some of these compounds have shown enhanced pharmacological profiles. This review is intended to describe our efforts to improve bioavailability of the aminopyridine derivatives, as well as to cover recent advances of other nNOS-selective inhibitors with promising pharmacological profiles.

DISCOVERY OF THE LEAD COMPOUNDS

In 2008 we reported a strategy called fragment hopping for the *de novo* design of nNOS-selective inhibitors.[20] Starting with crystal structures of nNOS complexed with peptide inhibitors, a series of 2-aminopyridinomethyl pyrrolidine analogues, including **1**, were designed and synthesized; the 2-aminopyridine moiety mimicked the guanidino group of L-arginine, and the pyrrolidine amino group replaced the primary amino group of L-arginine. Compound **1** had nanomolar potency (390 nM) with nNOS/eNOS and nNOS/iNOS selectivities of 1100 and 150, respectively. Up to here The crystal structure of nNOS complexed with **1** revealed that two steric or hydrophobic binding pockets could be accessed by attaching appropriate fragments. Subsequently, the racemic mixture of **2** was designed and synthesized, which had a K_i of 14 nM toward nNOS with a selectivity over eNOS of 2000 and over iNOS of 290. The selectivity was also reflected in animal experiments: ($\pm$)-**2** decreased the NO levels and NOS activity in the brains of newborn rabbit kits without affecting eNOS-regulated blood pressure.[21] The *in vitro* enzyme assay of the two *cis* enantiomers showed that the (3'*R*,4'*R*)-isomer (**4**) was more potent and more selective than the (3'*S*,4'*S*)-isomer (**3**) toward nNOS ($K_i = 5.3$ nM, 3830-fold over eNOS and 743-fold over iNOS).[22] To better understand structure-activity relationships, crystal structures of nNOS complexed with both of the enantiomers were determined. The (3'*S*,4'*S*)-isomer bound the same as **1** with hydrogen bonding of its 2-aminopyridine moiety to the NOS active site Glu and Trp residues, and the pyrrolidine nitrogen also interacting with this same Glu (Figure 1A). Surprisingly, (3'*R*,4'*R*)-isomer **4** exhibited a totally different binding mode; it flipped 180° from the orientation adopted by its enantiomer (Figure 1B). The 2-aminopyridine head makes bifurcated hydrogen bonds with heme propionate D, and the hydrophobic tail binds above the heme next to the active site Glu residue. Interestingly, there also is a new π -stacking interaction of the 2-aminopyridine with Tyr706, which contributes additional affinity.

Further modification of **4** gave **5**, which had a K_i of 7 nM and nNOS/eNOS and nNOS/iNOS selectivities of 2667 and 806, respectively.[23] The binding conformation of **5** was almost the same as that for **4**. The key structural determinant for their high selectivity of nNOS over eNOS was identified by crystallographic and site-directed mutagenesis studies: nNOS has two negative charges (Glu-592 and Asp-587) in the active site compared to one in eNOS (Glu-363), and, as a result, nNOS provides better electrostatic stabilization for the positively charged inhibitors, which is the primary reason why these inhibitors are so selective for nNOS.

Although **4** and **5** represent two of the most potent and selective nNOS inhibitors, they suffer from serious limitations, namely, the positive charges derived from the basic groups dramatically impair the ability of these inhibitors to penetrate the blood-brain barrier (BBB). The pyrrolidine N atom interacts with Asp-597 in nNOS, which is critical for nNOS over eNOS selectivity, so this moiety could not be altered. To improve the bioavailability of these inhibitors, we employed several strategies to circumvent the side chain positive charge of lead compounds **4** and **5**.

GEM-DIFLUORINATED MONOCATIONIC INHIBITORS

Fluorine is an exceptional atom that has been widely used to alter the pharmacokinetic properties and overall drug-like properties of compounds.[24] It is highly electron withdrawing and is lipophilic, while having the ability to protect metabolic sites; therefore, it has a significant effect on the pKa of neighboring groups, as well as having an effect on oral absorption, metabolism, and brain penetration of drugs. Incorporation of fluorine into molecules is a successful strategy, which has been utilized by many marketed fluorine-containing central nervous system agents.

To modulate the basicity of the side chain nitrogen atom of **5**, we introduced fluorine vicinal to the amino group in the hydrophobic tail; monofluoro ($\pm$)-**6** lowered the pKa of the amino group to 7.25, and gem-difluoro ($\pm$)-**7** lowered the pKa to 5.6, making the molecule essentially monocationic.[25,26] These compounds were only a little less potent and selective than the parent compound (**5**), having K_i values of 36 and 80 nM, respectively, with nNOS/eNOS selectivities of 1000-fold and 780-fold, respectively, and nNOS/iNOS selectivities of 360-fold and 650-fold, respectively. Syntheses of the two *cis* enantiomers of ($\pm$)-**7** were subsequently carried out; the (3'*R*,4'*R*)-isomer (**8**) had a K_i of 36 nM and nNOS/eNOS and nNOS/iNOS selectivities of 3800 and 1400, respectively. Its enantiomer had one-

tenth the potency with one-thirteenth and one-fourth the selectivities, respectively. Compounds ($\pm$)-**6**, ($\pm$)-**7**, and parent compound **5** were tested for their potency in a cell-based assay that provided information about their membrane permeability. Although ($\pm$)-**6** and ($\pm$)-**7** were only 0.9 and 0.47 times as potent as **5** in the cell-based assay, respectively, because of the much greater potency of **5** than **6** and **7** in the enzyme assay, these results indicate that **6** and **7** crossed the cell membrane 2.1 and 2.5 times *better* than **5**, respectively. Therefore, the *gem*-fluorinated monocationic inhibitor not only retained most of the activity of the lead compound, but also had improved membrane permeability. The pharmacokinetic properties of **8** were compared to those of **5** in rats, and it was found that **8** exhibited a much improved pharmacokinetic profile, having a half-life of 7.5 h for iv dosing at 1 mg/kg, a half-life of 3.7 h for oral dosing at 5 mg/kg, and measurable oral exposure with an estimated bioavailability of 22.2%. The corresponding half-life values for **5** were 0.33 h for iv dosing at 1 mg/kg, and too low to measure for oral dosing at 5 mg/kg, with no bioavailability.

INTRAMOLECULAR HYDROGEN BONDING

Incorporation of intramolecular hydrogen bonds into drug molecules is a useful strategy to mask the polarity of the molecules from the environment and thus improve their membrane permeability.[27] It is believed that the drug molecule adopts a ‘closed’ conformation as a result of formation of the intramolecular hydrogen bond in the hydrophobic environment of the membrane; when it reaches its target enzyme the pharmacologically critical polar group is exposed, allowing it to bind, which stabilizes the ‘open’ conformation.

We designed and synthesized four nNOS inhibitors to diffuse the overall charge by incorporation of an intramolecular hydrogen bond.[28] The phenylethyl tails, as in **3–5**, were replaced by benzyl tails with the aim to facilitate five- and six-membered ring intramolecular hydrogen bond formation (**9–12**). Cell permeability was estimated by the Relative Permeability Index (RPI), a ratio of the IC₅₀ value in the cellular assay versus the IC₅₀ value in the purified enzyme assay; the lower the RPI value, the better the cell permeability. All four compounds had lower potencies for nNOS (*K*_i values ranged from 390 to 820 nM) and lower selectivities over eNOS and iNOS (ranged from 93- to 169-fold and 92- to 105-fold, respectively) in comparison with the parent compounds. Nevertheless, as shown in Figure 2, **9** and **10** had higher cell permeability compared to **3**, and **11** was comparable in permeability relative to **3**.

MONOCATIONIC INHIBITORS WITH NEUTRAL SIDE CHAINS

Lipophilicity plays an important role in membrane permeability, as evidenced by the good permeability of **9** and the poor permeability of **11** (despite the fact that they exhibited almost same potencies for nNOS in the isolated enzyme assay). To enhance potential bioavailability, the lipophilicity of the compounds need to be increased and the charge decreased. Those aims led to the design of a new series of nNOS inhibitors in which the aminoalkyl tail of **5** was replaced by a lipophilic biphenyl fragment (see **13–16** for some examples).[29] All of these inhibitors were significantly less potent (300–600-fold) than

lead compound **5**, with K_i values in the low micromolar range, although the shape and size of the active site of nNOS were sufficient to fit this series of compounds on the basis of docking calculations. Compounds **13–16** showed slightly enhanced nNOS inhibition relative to others in this series, with K_i values ranging from 620 to 820 nM and nNOS/eNOS and nNOS/iNOS selectivities below 22-fold and 66-fold, respectively.

The most direct strategy to circumvent the side chain positive charge and further improve permeability would be removal of the side chain NH group. On the basis of this strategy, we synthesized a new series of monocationic nNOS inhibitors by replacement of the basic sidechain amino group of **5** with a methylene group.[30] These compounds also showed lowered potency and selectivities; for example, **17** had a K_i of 922 nM toward nNOS with selectivities over eNOS of 37 and over iNOS of 91. This compound was structurally characterized by X-ray crystallography of its complex with nNOS (Figure 3). Surprisingly, (3'*R*,4'*R*)-isomer **17** broke the precedent that had been established with many crystal structures, which demonstrated that the (3'*R*,4'*R*)-pyrrolidine analogues adopted a flipped binding mode similar to the conformation of **4** or **5**. Despite the (3'*R*,4'*R*) stereochemistry, **17** exhibited the unexpected normal binding mode similar to the conformation of (3'*S*,4'*S*)-isomer **3**. The 2-aminopyridine moiety interacted with Glu592 while the 3-fluorophenyl group extended out of the substrate-catalytic site and pointed toward the entrance of the binding pocket (Figure 3). The crystallographic structures of **17** and parent compound **5** in complex with nNOS demonstrated that even a small bioisosteric change, such as NH to CH₂, could cause important differences in the binding mode to an enzyme and consequently dramatically affect inhibitory potency. A steered molecular dynamics simulation provided some clues to explain the phenomenon.[33] It was found that the chemical nature of the side chain and the aromatic end group were two key factors that determined which way the inhibitors bind. The side chain and the fluorophenyl end of **17** were extremely unstable because of the lack of the side chain N atom, resulting in a curling of the tail prior to its much easier dissociation from the substrate-catalytic pocket. The simulation results implied that one or more heteroatoms embedded in the aromatic ring or linker chain should favorably stabilize the ligand by providing an additional hydrogen bond with the protein. On that basis, we synthesized a series of nNOS inhibitors (**18–21**) with the fluorophenyl tail of **17** replaced by pyridines that had the potential to form hydrogen bonding interactions with the enzyme, and importantly, would not be protonated under physiological conditions. Crystal structures of nNOS complexed with them showed that these inhibitors again adopted the flipped binding mode (Figure 4). Enzyme assay results indicated that they were potent and selective inhibitors toward nNOS, with K_i values in the low nanomolar range (from 30 to 74 nM). Remarkably, compound **18** was 2.7 times more potent than **7** in the cell-based nNOS assay, which demonstrated a successful improvement in membrane permeability.

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SYMMETRIC DOUBLE-HEADED AMINOPYRIDINES

As noted above, **3** bound to nNOS with its 2-aminopyridine motif above the heme next to the active site Glu, while the 2-aminopyridine motif of **4** made bifurcated hydrogen bonds with heme propionate D and p-stacked with Tyr706. This suggested that effective inhibition of nNOS might be achieved by double-headed 2-aminopyridine derivatives with the appropriate linker. Once the basic amino groups in the parent compound were replaced with a hydrophobic linker, a significant increase in lipophilicity could potentially improve membrane permeability. Consequently, a series compounds, including **22**, was synthesized and tested for inhibition of the three isozymes.[31] Most of these compounds exhibited submicromolar binding affinity to nNOS and modest selectivity against eNOS and iNOS. The best inhibitor in this series, **22**, had a K_i of 25 nM and nNOS/eNOS and nNOS/iNOS selectivities of 107 and 58, respectively. Crystal structures of **22** bound to nNOS showed that it binds as designed with one aminopyridine interacting with Glu592 and the other interacting with the heme propionate D (Figure 5).[32] Interestingly, a second molecule of **22** also bound to nNOS with one aminopyridine head displacing the tetrahydrobiopterin cofactor; none of this occurred in eNOS, which accounted for the 107-fold selectivity for nNOS over eNOS. Remarkably, **22** showed good membrane permeability, with an IC_{50} value in the cell-based assay of 5 μ M, which was significantly better than that of lead compound **5**.

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THIOPHENE-2-CARBOXIMIDAMIDE DERIVATIVES

A thiophene-2-carboximidamido fragment could provide an effective mimic for the guanidinium group of the natural NOS substrate L-Arg, as was the case for the 2-aminopyridine of the compounds described above. The carboximidamide nitrogen atoms could serve as H-bond acceptors or H-bond donors, forming hydrogen bonds with the

Glu592 side chain and the Trp587 main chain oxygen atom, respectively; the thiophene sulfur atom could form van der Waals contacts with the porphyrin, leading to strong multiple-point interactions with the enzyme. The early milestone in this series of nNOS-selective inhibitors was the discovery of **23** by AstraZeneca.[33] This compound bound most tightly ($IC_{50} = 35$ nM) to nNOS, and about one and two orders of magnitude more weakly to iNOS and eNOS, respectively. The main stabilizing interactions observed in the nNOS-**23** complex are shown in Figure 6.[34] The thiophene-2-carboximidamide motif bound to the nNOS active site as was described above for the 2-aminopyridine analogues. The amino group formed a hydrogen bond with heme propionate D, while the chlorophenyl group fit nicely into a hydrophobic pocket defined by residues Tyr706, Trp678, Met336, and Leu337 and stabilized by stacking interactions with the aromatic ring of Tyr706. However, unlike the 2-aminopyridine analogues, **23** could rapidly penetrate the rat brain and produce effective and long-lasting inhibition of nNOS *in vivo*; following a single 10 mg/kg iv dose of **23** in rats, cerebellar nNOS was inhibited by 70% at 2 h and was still inhibited by 50% at 24 h, as assessed by ex vivo analysis.[33]

Because of their excellent nNOS inhibitory properties and pharmacological profiles, thiophene-2-carboximidamide based inhibitors were investigated by means of kinetic, physiological, and pharmacological studies over the last decade. A dozen active analogues with promising pharmacological profiles emerged during the past two years. For example, Sanofi-Aventis reported a series of inhibitors with an amidinothiophene hydroxypiperidine scaffold (e.g., **24–26**), with the aim of projecting a part of the inhibitor into the selectivity modulating region of nNOS, thereby leveraging unique hydrophobic interactions with residues in this area.[35] Inhibitors with a chlorophenyl tail exhibited good potency for nNOS and moderate to high selectivity against eNOS. In this case, IC_{50} values of 131, 129, and 17 nM for **24**, **25**, and **26**, respectively, were measured. Compound **26** showed a significant 1664-fold selectivity for nNOS over eNOS. However, it had low metabolic stability with only 68% remaining after incubation with purified human liver microsomes for 20 minutes.

Recently, NeurAxon reported numerous thiophene-2-carboximidamide analogues in which some were potent and selective, and with remarkably good oral bioavailability. Initially, a series of 3,4-dihydroquinolin-2(1*H*)-ones containing a 6-substituted thiophene amidine

group (e.g., **27**) were synthesized and evaluated as inhibitors of nNOS.[36] A number of different basic amino side chains were attached to the amide nitrogen atom. Enzyme assay results indicated that most of them showed moderate potency (with IC_{50} values in the low micromolar range) and low selectivity (less than 200-fold and 130-fold for nNOS/eNOS and nNOS/iNOS, respectively). The best one, **27**, had an IC_{50} of 160 nM toward nNOS with selectivities over eNOS of 181 and over iNOS of 69. Subsequently, many 1,2,3,4-tetrahydroquinolines, which are structural analogues of 3,4-dihydroquinolin-2(1H)-ones, were synthesized and investigated for their selective nNOS inhibitory action (**28–31**).[36] In this series, compounds with 2- and 3-carbon acyclic linkers showed submicromolar activities against nNOS. The most selective compound, **28**, (465-fold and 92-fold for nNOS/eNOS and nNOS/iNOS, respectively) was also one of the most potent nNOS inhibitors (IC_{50} = 98 nM), while its enantiomer (**29**) was less selective against nNOS over eNOS with an IC_{50} value of 130 nM for nNOS. Interestingly, **28** possessed favorable pharmacological properties; it was shown to fully reverse thermal hyperalgesia when given to rats at a dose of 30 mg/kg intraperitoneally in the L₅/L₆ spinal nerve ligation model of neuropathic pain (the Chung model). Furthermore, it reduced tactile hyperesthesia (allodynia) after oral administration (30 mg/kg) in a rat model of dural inflammation relevant to migraine pain. However, **28** suffered from low oral bioavailability (only 18%) and had moderate hERG channel inhibition (IC_{50} = 4.7 μ M). Therefore, modifications focused on the substituent on 1,2,3,4-tetrahydroquinoline nitrogen atom were subsequently performed to optimize the properties of **28**. [37] Compound **30** was identified as a potent (nNOS IC_{50} = 176 nM) and selective (231-fold and more than 500-fold for nNOS/eNOS and nNOS/iNOS, respectively) nNOS inhibitor with improved oral bioavailability (up to 60%) and weak hERG channel inhibition (IC_{50} > 30 μ M). Expansion of the aliphatic cycle in 1,2,3,4-tetrahydroquinoline led to a class of 1,7-disubstituted 2,3,4,5-tetrahydro-1H-benzo[b]azepine-based thiophene amidines.[38] The effect of various basic amino side chains attached at the 1-position was investigated. Compound **31** was an interesting member of this class, which was an efficient inhibitor of human nNOS (K_i value of 80 nM) with very good selectivities over eNOS and iNOS (756-fold and more than 1250-fold, respectively).

In addition to the quinoline analogues, indole and indoline derivatives have been among the most structurally studied heterocyclic scaffolds for the discovery of nNOS inhibitors. Many compounds in this class (for example, **32–36**) exhibited submicromolar nNOS inhibition (Figure 7), but suffered from low selectivities, especially over eNOS.[39–44] However, compounds **32–36** were shown to reverse thermal hyperalgesia *in vivo* in the L₅/L₆ spinal nerve ligation model of neuropathic pain. Although **32** only had a nNOS/eNOS selectivity of 33-fold, it was devoid of any significant vasoconstrictive effects in human coronary arteries, which is associated with the inhibition of human eNOS. 3,5-Disubstituted indole derivative **33** was one of the most interesting compounds of this class because it exhibited dual nNOS and norepinephrine transporter inhibition (IC_{50} of 0.56 and 1.0 μ M, respectively). It also showed a lack of any direct vasoconstriction or inhibition of Acetylcholine-mediated vasorelaxation in isolated human coronary arteries. The *in vivo* results made these indole/

indoline derivatives promising nNOS inhibitors that can mitigate the cardiovascular liabilities associated with the higher lipophilic tetrahydroquinoline-based nNOS inhibitors.

CONCLUSIONS AND FUTURE OUTLOOK

A variety of pyrrolidine-based aminopyridine derivatives exhibit excellent potencies and isoform selectivities and some represent the most selective in vitro nNOS inhibitors to date. However, their therapeutic potential for the treatment of neurodegenerative diseases is still limited because of unfavorable pharmacokinetics, even though some successful improvements have been achieved by structural modifications. On the other hand, some thiophene-2-carboximidamide derivatives showed much more interesting pharmacological profiles compared to the aminopyridines, but they have significant challenges to overcome with respect to their lower potency and isozyme selectivities in vitro. Few crystal structures depicting the binding mode of these molecules to the NOS isoforms have been reported; more crystallographic studies of these compounds would be highly desirable.

Both the 2-aminopyridine and thiophene-2-carboximidamide moieties exhibit similar hydrogen bond interactions with the enzyme, acting as mimics of the guanidinium group of the natural substrate L-Arg. Since the former class exhibits excellent potency and isoform selectivities, and the latter class possesses promising pharmacological profiles, hybrid chemical structures combining the thiophene-2-carboximidamide head and structural features of known potent and selective aminopyridine inhibitors would be desirable. Such a strategy might result in potent and selective drug-like molecules with enhanced pharmacological properties that are more effective than the parent molecules.

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LIST OF ABBREVIATIONS

NO	Nitric oxide
NOS	Nitric oxide synthase
nNOS	Neuronal nitric oxide synthase
eNOS	Endothelial neuronal nitric oxide synthase
iNOS	Inducible neuronal nitric oxide synthase
NADPH	Reduced nicotinamide adenine dinucleotide phosphate
NADP⁺	Nicotinamide adenine dinucleotide phosphate
BBB	Blood-brain barrier
RPI	Relative Permeability Index

References

1. Alderton WK, Cooper CE, Knowles RG. Nitric oxide synthases: Structure, function and inhibition. *Biochem J.* 2001; 357:593–615. [PubMed: 11463332]
2. Vallance P, Leiper J. Blocking NO synthesis: How, where and why? *Nat Rev Drug Discov.* 2002; 1:939–950. [PubMed: 12461516]

3. Rosen GM, Tsai P, Pou S. Mechanism of free-radical generation by nitric oxide synthase. *Chem Rev.* 2002; 102:1191–1199. [PubMed: 11942793]
4. Olesen J. Nitric oxide-related drug targets in headache. *Neurotherapeutics.* 2010; 7:183–190. [PubMed: 20430317]
5. Villanueva C, Giulivi C. Subcellular and cellular locations of nitric oxide synthase isoforms as determinants of health and disease. *Free Radical Bio Med.* 2010; 49:307–316. [PubMed: 20388537]
6. Aquilano K, Baldelli S, Rotilio G, Ciriolo MR. Role of nitric oxide synthases in Parkinson's disease: A review on the antioxidant and anti-inflammatory activity of polyphenols. *Neurochem Res.* 2008; 33:2416–2426. [PubMed: 18415676]
7. Masic LP. Arginine mimetic structures in biologically active antagonists and inhibitors. *Curr Med Chem.* 2006; 13:3627–3648. [PubMed: 17168727]
8. Oess S, Icking A, Fulton D, Govers R, Muller-Esterl W. Subcellular targeting and trafficking of nitric oxide synthases. *Biochem J.* 2006; 396:401–409. [PubMed: 16722822]
9. Silverman RB. Design of selective neuronal nitric oxide synthase inhibitors for the prevention and treatment of neurodegenerative diseases. *Accounts Chem Res.* 2009; 42:439–451.
10. Maddaford S, Annedi SC, Ramnauth J, Rakhit S. Advancements in the development of nitric oxide synthase inhibitors. *Annu Rep Med Chem.* 2009; 44:27–50.
11. Hesslinger C, Strub A, Boer R, Ulrich WR, Lehner MD, Braun C. Inhibition of inducible nitric oxide synthase in respiratory diseases. *Biochem Soc Trans.* 2009; 37:886–891. [PubMed: 19614613]
12. Kanwar JR, Kanwar RK, Burrow H, Baratchi S. Recent advances on the roles of NO in cancer and chronic inflammatory disorders. *Curr Med Chem.* 2009; 16:2373–2394. [PubMed: 19601787]
13. Granik VG, Grigor'ev NA. Nitric oxide synthase inhibitors: Biology and chemistry. *Russ Chem Bull.* 2002; 51:1973–1995.
14. Auvin S, Auguet M, Navet E, Harnett JJ, Viossat I, Schulz J, Bigg D, Chabrier PE. Novel inhibitors of neuronal nitric oxide synthase with potent antioxidant properties. *Bioorg Med Chem Lett.* 2003; 13:209–212. [PubMed: 12482425]
15. Beaton H, Boughton-Smith N, Hamley P, Ghelani A, Nicholls DJ, Tinker AC, Wallace AV. Thienopyridines: Nitric oxide synthase inhibitors with potent in vivo activity. *Bioorg Med Chem Lett.* 2001; 11:1027–1030. [PubMed: 11327581]
16. Ueda S, Terauchi H, Yano A, Ido M, Matsumoto M, Kawasaki M. 4,5-disubstituted-1,3-oxazolidin-2-imine derivatives: A new class of orally bioavailable nitric oxide synthase inhibitor. *Bioorg Med Chem Lett.* 2004; 14:313–316. [PubMed: 14698148]
17. Collins JL, Shearer BG, Oplinger JA, Lee SL, Garvey EP, Salter M, Duffy C, Burnette TC, Furfine ES. *N*-Phenylamidines as selective inhibitors of human neuronal nitric oxide synthase: Structure-activity studies and demonstration of in vivo activity. *J Med Chem.* 1998; 41:2858–2871. [PubMed: 9667974]
18. Aoki S, Ye Y, Higuchi K, Takashima A, Tanaka Y, Kitagawa I, Kobayashi M. Novel neuronal nitric oxide synthase (nNOS) selective inhibitor, aplysinopsin-type indole alkaloid, from marine sponge *hyrtios erecta*. *Chem Pharm Bull.* 2001; 49:1372–1374. [PubMed: 11605676]
19. Palumbo A, d'Ischia M, Cioffi FA. 2-Thiouracil is a selective inhibitor of neuronal nitric oxide synthase antagonising tetrahydrobiopterin-dependent enzyme activation and dimerisation. *FEBS Lett.* 2000; 485:109–112. [PubMed: 11094150]
20. Ji H, Stanton BZ, Igarashi J, Li H, Martasek P, Roman LJ, Poulos TL, Silverman RB. Minimal pharmacophoric elements and fragment hopping, an approach directed at molecular diversity and isozyme selectivity. Design of selective neuronal nitric oxide synthase inhibitors. *J Am Chem Soc.* 2008; 130:3900–3914. [PubMed: 18321097]
21. Ji H, Tan S, Igarashi J, Li H, Derrick M, Martasek P, Roman LJ, Vasquez-Vivar J, Poulos TL, Silverman RB. Selective neuronal nitric oxide synthase inhibitors and the prevention of cerebral palsy. *Ann Neurol.* 2009; 65:209–217. [PubMed: 19235180]
22. Delker SL, Ji H, Li H, Jamal J, Fang J, Xue F, Silverman RB, Poulos TL. Unexpected binding modes of nitric oxide synthase inhibitors effective in the prevention of a cerebral palsy phenotype in an animal model. *J Am Chem Soc.* 2010; 132:5437–5442. [PubMed: 20337441]

23. Ji H, Delker SL, Li HY, Martasek P, Roman LJ, Poulos TL, Silverman RB. Exploration of the active site of neuronal nitric oxide synthase by the design and synthesis of pyrrolidinomethyl 2-aminopyridine derivatives. *J Med Chem*. 2010; 53:7804–7824. [PubMed: 20958055]
24. Hodgetts KJ, Combs KJ, Elder AM, Harriman GC. The role of fluorine in the discovery and optimization of cns agents: Modulation of drug-like properties. *Ann Rep Med Chem*. 2010; 45:429–448.
25. Xue F, Fang J, Lewis WW, Martasek P, Roman LJ, Silverman RB. Potent and selective neuronal nitric oxide synthase inhibitors with improved cellular permeability. *Bioorg Med Chem Lett*. 2010; 20:554–557. [PubMed: 19963381]
26. Xue F, Li H, Delker SL, Fang J, Martasek P, Roman LJ, Poulos TL, Silverman RB. Potent, highly selective, and orally bioavailable gem-difluorinated monocationic inhibitors of neuronal nitric oxide synthase. *J Am Chem Soc*. 2010; 132:14229–14238. [PubMed: 20843082]
27. Kuhn B, Mohr P, Stahl M. Intramolecular hydrogen bonding in medicinal chemistry. *J Med Chem*. 2010; 53:2601–2611. [PubMed: 20175530]
28. Jansen Labby K, Xue F, Kraus JM, Ji H, Mataka J, Li H, Martasek P, Roman LJ, Poulos TL, Silverman RB. Intramolecular hydrogen bonding: A potential strategy for more bioavailable inhibitors of neuronal nitric oxide synthase. *Bioorg Med Chem*. 2012; 20:2435–2443. [PubMed: 22370337]
29. Xue F, Huang J, Ji H, Fang J, Li H, Martasek P, Roman LJ, Poulos TL, Silverman RB. Structure-based design, synthesis, and biological evaluation of lipophilic-tailed monocationic inhibitors of neuronal nitric oxide synthase. *Bioorg Med Chem*. 2010; 18:6526–6537. [PubMed: 20673724]
30. Huang H, Ji H, Li H, Jing Q, Jansen Labby K, Martasek P, Roman LJ, Poulos TL, Silverman RB. Selective monocationic inhibitors of neuronal nitric oxide synthase. Binding mode insights from molecular dynamics simulations. *J Am Chem Soc*. 2012; 134:11559–11572. [PubMed: 22731813]
31. Xue F, Fang J, Delker SL, Li H, Martasek P, Roman LJ, Poulos TL, Silverman RB. Symmetric double-headed aminopyridines, a novel strategy for potent and membrane-permeable inhibitors of neuronal nitric oxide synthase. *J Med Chem*. 2011; 54:2039–2048. [PubMed: 21410186]
32. Delker SL, Xue F, Li H, Jamal J, Silverman RB, Poulos TL. Role of zinc in isoform-selective inhibitor binding to neuronal nitric oxide synthase. *Biochem*. 2010; 49:10803–10810. [PubMed: 21138269]
33. Reif DW, McCarthy DJ, Cregan E, Macdonald JE. Discovery and development of neuronal nitric oxide synthase inhibitors. *Free Radical Bio Med*. 2000; 28:1470–1477. [PubMed: 10927171]
34. Fedorov R, Vasan R, Ghosh DK, Schlichting I. Structures of nitric oxide synthase isoforms complexed with the inhibitor AR-R17477 suggest a rational basis for specificity and inhibitor design. *Proc Natl Acad Sci USA*. 2004; 101:5892–5897. [PubMed: 15071192]
35. Liang G, Neuenschwander K, Chen X, Wei L, Munson R, Francisco G, Scotese A, Shutske G, Black M, Sarhan S, Jiang J, Morize I, Vaz RJ. Structure-based design, synthesis, and profiling of potent and selective neuronal nitric oxide synthase (nNOS) inhibitors with an amidinothiophene hydroxypiperidine scaffold. *MedChemComm*. 2011; 2:201–205.
36. Ramnauth J, Speed J, Maddaford SP, Dove P, Annedi SC, Renton P, Rakhit S, Andrews J, Silverman S, Mladenova G, Zinghini S, Nair S, Catalano C, Lee DK, De Felice M, Porreca F. Design, synthesis, and biological evaluation of 3,4-dihydroquinolin-2(1*H*)-one and 1,2,3,4-tetrahydroquinoline-based selective human neuronal nitric oxide synthase (nNOS) inhibitors. *J Med Chem*. 2011; 54:5562–5575. [PubMed: 21699209]
37. Ramnauth J, Renton P, Dove P, Annedi SC, Speed J, Silverman S, Mladenova G, Maddaford SP, Zinghini S, Rakhit S, Andrews J, Lee DK, Zhang D, Porreca F. 1,2,3,4-Tetrahydroquinoline-based selective human neuronal nitric oxide synthase (nNOS) inhibitors: Lead optimization studies resulting in the identification of n-(1-(2-(methylamino)ethyl)-1,2,3,4-tetrahydroquinolin-6-yl)thiophene-2-carboximi damide as a preclinical development candidate. *J Med Chem*. 2012; 55:2882–2893. [PubMed: 22335555]
38. Annedi SC, Ramnauth J, Cossette M, Maddaford SP, Dove P, Rakhit S, Andrews JS, Porreca F. Novel, druglike 1,7-disubstituted 2,3,4,5-tetrahydro-1*H*-benzo[*b*]azepine-based selective inhibitors of human neuronal nitric oxide synthase (nNOS). *Bioorg Med Chem Lett*. 2012; 22:2510–2513. [PubMed: 22370270]

39. Renton P, Speed J, Maddaford S, Annedi SC, Ramnauth J, Rakhit S, Andrews J. 1,5-Disubstituted indole derivatives as selective human neuronal nitric oxide synthase inhibitors. *Bioorg Med Chem Lett*. 2011; 21:5301–5304. [PubMed: 21813276]
40. Annedi SC, Maddaford SP, Mladenova G, Ramnauth J, Rakhit S, Andrews JS, Lee DKH, Zhang DQ, Porreca F, Bunton D, Christie L. Discovery of N-(3-(1-methyl-1,2,3,6-tetrahydropyridin-4-yl)-1*H*-indol-6-yl) thiophene-2-carboximidamide as a selective inhibitor of human neuronal nitric oxide synthase (nNOS) for the treatment of pain. *J Med Chem*. 2011; 54:7408–7416. [PubMed: 21923116]
41. Mladenova G, Annedi SC, Ramnauth J, Maddaford SP, Rakhit S, Andrews JS, Zhang DQ, Porreca F. First-in-class, dual-action, 3,5-disubstituted indole derivatives having human nitric oxide synthase (nNOS) and norepinephrine reuptake inhibitory (neri) activity for the treatment of neuropathic pain. *J Med Chem*. 2012; 55:3488–3501. [PubMed: 22420844]
42. Maddaford S, Renton P, Speed J, Annedi SC, Ramnauth J, Rakhit S, Andrews J, Mladenova G, Majuta L, Porreca F. 1,6-Disubstituted indole derivatives as selective human neuronal nitric oxide synthase inhibitors. *Bioorg Med Chem Lett*. 2011; 21:5234–5238. [PubMed: 21824773]
43. Annedi SC, Maddaford SP, Ramnauth J, Renton P, Speed J, Rakhit S, Andrews JS, Porreca F. 3,5-Disubstituted indole derivatives as selective human neuronal nitric oxide synthase (nNOS) inhibitors. *Bioorg Med Chem Lett*. 2012; 22:1980–1984. [PubMed: 22318159]
44. Annedi SC, Ramnauth J, Maddaford SP, Renton P, Rakhit S, Mladenova G, Dove P, Silverman S, Andrews JS, Felice MD, Porreca F. Discovery of *cis*-N-(1-(4-(methylanino)cyclohexyl)indolin-6-yl)thiophene-2-carboximidamide: A 1,6-disubstituted indoline derivative as a highly selective inhibitor of human neuronal nitric oxide synthase (nNOS) without any cardiovascular liabilities. *J Med Chem*. 2012; 55:943–955. [PubMed: 22175766]

Fig. 1.
Crystallographic binding conformations of **(A)** **3** (PDB ID 3JWS) and **(B)** **4** (PDB ID 3JWT) with rat nNOS.

Fig. 2.
Relative Permeability Indices (RPI) of **3** and **9–12**.

Fig. 3.
Crystallographic binding conformation of **17** with rat nNOS (PDB ID 3UFP).

Fig. 4. Crystallographic binding conformations of (A) **19** (PDB ID 3UFV) and (B) **20** (PDB ID 3UFW) with rat nNOS.

Fig. 5.
Crystallographic binding conformation of **22** (PDB ID 3N5W) with rat nNOS.

Fig. 6.
Crystallographic binding conformation of **23** (PDB ID 1VAG) with rat nNOS.

Fig. 7.
Inhibition of human nNOS by **32–36**.