Chapter 4

Review 3: Novel Nitric Oxide Synthase (NOS) Inhibitors: a Patent Review

Article published online on 22 February 2011

Expert Opinion on Therapeutic Patents 21:4 (2011) 537-560

Novel Nitric Oxide Synthase (NOS) Inhibitors: a Patent Review

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Article Highlights

- NOS catalyzes the production of NO, an important cellular signaling molecule with a pivotal role in many biological processes, and is currently under scrutiny as a potential therapeutic target in the treatment of rheumatoid arthritis, inflammation, pain and neurodegenerative diseases.
- Research attention and expenditure are focused on iNOS and nNOS due to their involvement in various pathological conditions.
- The physiological role of eNOS has precluded this isoform as drug target.
- Recent patents of structures with potent and selective inhibition of the selected NOS isoforms favor extended heterocyclic structures, incorporating guanidine-like moieties or bioisosters thereof.

Abstract

Introduction: Knowledge of nitric oxide and its function in cell signaling has rapidly developed since its first biological effects were described in 1977. It is formed from *L*-arginine by nitric oxide synthase isoforms (nNOS, iNOS and eNOS). These enzymes are products of separate genes, encoded on three different chromosomes and responsible for regulating a variety of functions within cells and tissues, which include vasodilatation, neurotransmission and the immunological process. NOS isoforms are currently under investigation as targets for novel therapeutics in especially neurodegenerative disorders, inflammation and pain. Many important questions regarding the formation, function and metabolism of these important messengers and signaling molecules remain to be answered.

Areas covered in this review: This review gives an overview of patents covering drug-like inhibitors for the NOS isoforms filed and published within the last 6 years, up to September 2010, as well as insight into recent highlights in this area.

Expert opinion: The NOS isoforms are attractive targets in drug design for various pathological conditions and have received considerable interest over recent years. With the advances in molecular biology, modeling software, synthesis, bioassays and our understanding of the NOS enzymes and the function of NO, novel bioavailable and highly selective drug therapies utilizing this mode of action may soon see the light.

Keywords: Drug Discovery, NOS Inhibitors, NOS Isoforms, Therapeutic Applications.

4.1. Introduction

The role of nitric oxide (NO) as a biological signaling molecule and its diverse functions in normal and pathological processes are well established [1]. These include the regulation of blood pressure, neurotransmission and controlling the macrophage defense systems [2]. NO is synthesized by nitric oxide synthases (NOS), a class of homodimeric heme proteins that catalyze an NADPH- (nicotinamide adenine dinucleotide phosphate) and O_2-dependant five electron oxidation of *L*-arginine to *L*-citrulline and NO *via* the intermediate N^G-hydroxy-*L*-arginine (Figure 1) [3, 4]. The NOS isoforms are the only known enzymes that have several co-factors essential for its activity, including NADPH, flavin adenine dinucleotide (FAD), flavin mononucleotide (FMN), heme, tetrahydrobiopterin (BH_4) and calmodulin [5] (Figure 2).

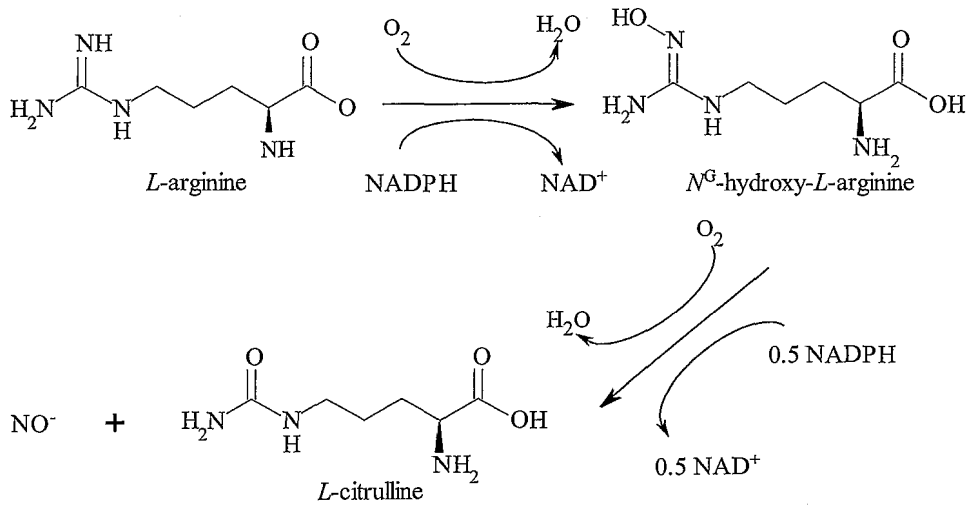


Figure 1: The general scheme for the biosynthesis of NO from *L*-arginine

Three distinct NOS enzymes have been identified and characterized in mammals [6], products of different genes, with different sub cellular localisation, regulation, catalytic properties, and inhibitor sensitivity. The three NOS isoforms show 50-60% sequence homology (Figure 2), and are associated with different physiological functions: neuronal nitric oxide synthase (nNOS) generates NO in the central nervous system (CNS) and is involved in neurotransmission and long-term potentiation; endothelial nitric oxide synthase (eNOS) derived NO is involved in the regulation of smooth muscle relaxation and vascular tone; and inducible nitric oxide synthase (iNOS), in macrophages, is important in the immune system's defense against pathogens and tumorous cells [7]. NO has also been linked to guanile cyclase activation, neurotransmitter release and reuptake, as well as glutamate (NMDA) mediated neurotoxicity [8] leading to apoptosis.

All three isoforms are active as homodimers with each subunit containing a C-terminal reductase domain (with binding sites for NADPH and the flavins FAD and FMN) and an N-terminal oxygenase domain containing a heme prostetic group (Figure 2). The C-terminal reductase domain, homologous to cytochrome P_{450} reductase, has binding sites for the substrate *L*-arginine, heme and the redox cofactor, biopterin (BH_4). The C-terminal reductase and N-terminal oxygenase domains are linked in the middle of the protein to a calmodulin-binding domain [9]. Binding of calmodulin, regulated by intracellular calcium, appears to act as a molecular switch to enable electron flow from the flavin prosthetic groups in the reductase domain to the heme group. This facilitates the conversion of oxygen and *L*-arginine to NO and *L*-citrulline [10]. The BH_4 prosthetic group, is required for efficient generation of NO. Unlike other enzymes where BH_4 is used as a source of reducing equivalents and is

recycled by dihydrobiopterin reductase, BH_4 activates heme-bound oxygen by donating a single electron, which is then recaptured to enable nitric oxide release [11]. Inhibition of NOS activity, and subsequently NO production, can be achieved by binding of the NOS inhibitors to one or both of the monomers. Inhibition of iNOS can further be achieved by inhibition of dimerization to form the biologically active dimeric enzyme [12].

nNOS and eNOS are constitutively expressed and physiologically activated by steroid hormones or neurotransmitters such as NO, dopamine, glutamate and glycine that increase intracellular calcium concentrations, leading to intermittent production of small amounts of NO [5]. In contrast, iNOS is calcium independent and is expressed in a broad range of cell types. This form of NOS is induced after stimulation by cytokines and exposure to microbial products, which leads to the production of large amounts of NO [6]. The constitutive enzymes are dependent on the physiological concentration of calcium in cells that regulate the binding of calmodulin to the "latch domains", thereby initiating electron transfer from the NADPH *via* FAD and FMN to the heme moieties. Calmodulin, however, remains tightly bound to the inducible and calcium-insensitive isoform (iNOS) even at a low intracellular calcium concentration, acting essentially as a subunit that activates this isoform [6, 9, 13].

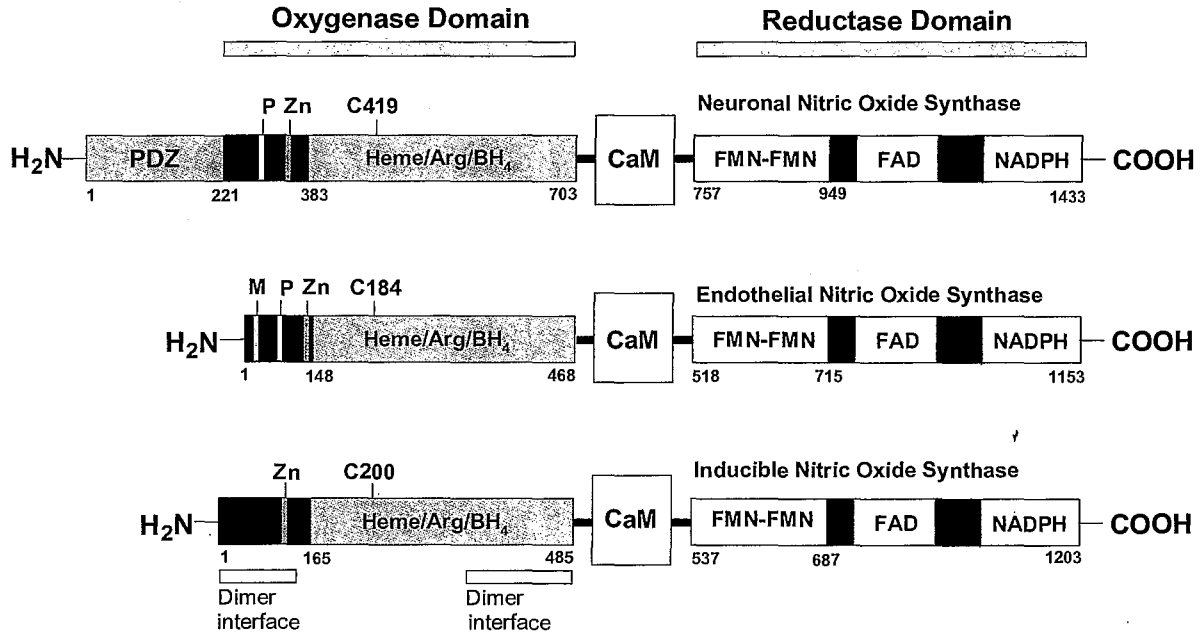


Figure 2. An illustration of the domain structure and amino acid sequence of the human NOS isoforms. The PDZ, oxygenase, and reductase domains and amino acid residue number at the start/end of each domain is shown. Myristoylation (M) site on eNOS and the palmitoylation (P) sites on eNOS and iNOS are shown, as is the location of the zinc-ligating cysteines (Zn). The cysteine residues (C419, C184, C200) which ligates the heme and the CaM-binding site is indicated for each isoform. All NOS isoforms contain a region in the oxygenase domain that includes the binding sites for heme,

L-arginine (Arg), and tetrahydrobiopterin (BH_4). The white bars indicate the dimer interface in the oxygenase domain and the oxygenase and reductase domains are separated by the calmodulin binding region (CaM). The reductase domain contains binding sites for flavin mononucleotide (FMN) and flavin-adenine dinucleotide (FAD) as well as several consensus sites for the electron donor species nicotinamideadenine dinucleotide phosphate (NADPH). [5, 12].

NO may itself regulate NOS expression and activity and has specifically been shown to play an important negative feedback regulatory role on eNOS, and therefore vascular endothelial cell function [14]. This process, known as *S*-nitrosation (and referred to by many in the field as *S*-nitrosylation), has been shown to reversibly inhibit eNOS activity in vascular endothelial cells. This process may be important as it is regulated by cellular redox conditions and may thereby provide a mechanism for the association between oxidative stress and endothelial dysfunction [14]. In addition to eNOS, both nNOS and iNOS can be *S*-nitrosated, but the evidence for dynamic regulation of these NOS isoforms by this process is less complete [15]. Both nNOS and eNOS have also been shown to form ferrous-nitrosyl complexes on their heme prosthetic groups that may act partially to self-inactivate these enzymes under certain conditions. The rate-limiting step for the production of nitric oxide may well still be the availability of *L*-arginine in some cell types. This may be particularly important after the induction of iNOS [12,16].

Regulation of nNOS expression is complex and occurs through alternative splicing, deletion and insertion of exons and multiple promoter usage [17]. Post-transcriptional regulation of the nNOS gene occurs in the form of alternative mRNA splicing and the gene products of four nNOS splice variants that have been detected so far include nNOS-μ, nNOS-α, nNOS-γ and nNOS-ß. As yet, the determinants of alternative-splicing events or how these events are regulated and the biological significance of the nNOS splice variants are poorly understood [18]. Antisense studies have suggested that the splice variants of nNOS play pharmacologically distinct roles. nNOS-μ is selectively expressed in rat heart and is the predominant isoform in rat skeletal muscle [19], and in rat and human penis and urethra [20]. It has an additional 34 amino acids inserted between the calmodulin-binding region and flavin-binding domains and is the most extensively characterized of the nNOS splice variants. An mRNA variant of nNOS was also detected in mouse brain that, if translated, would result in a 104-amino-acid in-frame deletion of residues 504 - 608 [21]. This splice variant has been called nNOS-α and detected in human neuroblastoma cell lines [22]. The deletion in this highly conserved region of nNOS that is critical for L-arginine binding has led to speculation that nNOS-α may be catalytically inactive and may, therefore, function as a dominant

negative regulator of nNOS activity [23]. nNOS-ß and nNOS-γ, which lack the membrane PDZ-binding domain of nNOS, have been identified but their functions are not yet clear. The latter isoforms are expressed at low levels in brain tissue containing nNOS and contribute to residual nNOS catalytic activity [24]. The presence of the PDZ domain in the enzymes determines specific binding sites for protein inhibition of NOS [25], the NMDA receptor [26] and syntropin [27]. These protein-protein interactions may regulate NO production and signal transduction. Relatively little information on iNOS splice variants is available and as yet no splice variants of eNOS have been described.

Although NO mediates several physiological functions, a number of disease states are associated with either the overproduction of, or overstimulation by NO, making the NOS pathway an attractive target for the development of therapeutics [3]. Overstimulation of individual NOS isoforms, especially nNOS and iNOS, plays a role in several disorders, including septic shock, arthiritis [28], diabetes, ischemia-reperfusion injury, pain [4], and various neurodegenerative diseases [2]. In contrast, NO produced by eNOS in endothelial cells, has mainly a physiological role, such as maintaining normal blood pressure and flow [29] and the inhibition thereof leads to unwanted effects such as enhanced white cell and platelet activation, hypertension and increased atherogenesis [30].

The therapeutic benefit of agents that decrease NO levels is thus controversial as NO may exert both positive and negative effects on physiological conditions and pathophysiological progression [31]. The selective inhibition of nNOS or iNOS, but not of eNOS, could however provide effective therapeutic approaches [32] and selective inhibitors could also be useful tools for investigating other biological functions of NO [33].

Because free NO is a transient species with a half life of about five seconds, many investigations of this gaseous molecule have relied largely on studies of the NOS enzymes [8]. A common pharmacological approach to study the role of a biological mediator is to investigate how processes related to it are affected by the administration of specific inhibitors. As a result, guanidines such as *L*-NAME (**1**), *L*-NMMA (**2**) and aminoguanidine (**3**) (Figure 3) are NOS inhibitors used extensively in pharmacological tests, where it is believed that their pharmacological effects are exerted by inhibition of NOS isoforms and a presumed decreased NO concentration in tissues [34, 35]. Since the isoforms possess a distinct cellular localization and are differently regulated, they represent specific targets for potential therapeutic approaches. Several reviews focusing on recent pharmacological updates on the

NOS isoforms have been published, serving as a good starting point for further exploration [1, 2, 6, 10, 12, 31, 33].

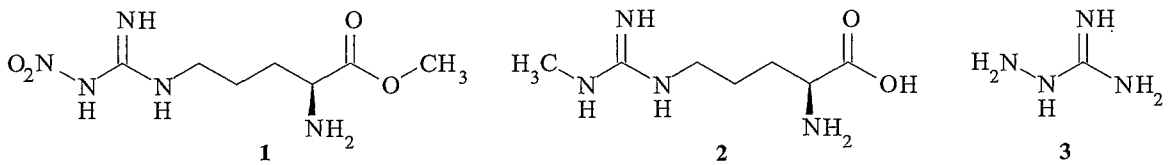


Figure 3: Guanidine derivatives as NOS inhibitors

The following sections discuss the disease implications of each of the three NOS isoforms, providing brief pharmacological information and known ligands with the focus on recently patented compounds.

4.2. Recent patents on NOS isoform inhibitors

This section is presented based on the three NOS isoforms (nNOS, iNOS and eNOS) and their respective inhibitors, either being isoform selective or nonselective.

4.2.1. Neuronal Nitric Oxide Inhibitors

nNOS catalyzes the oxidation of *L*-arginine to *L*-citrulline in the central nervous system, generating NO, a critical neurotransmitter [30]. Research has implicated the overexpression of nNOS – and overproduction of NO – in various neurological diseases, which includes amongst others, Parkinson's, Alzheimer's and Huntington's diseases, as well as neuronal damage due to stroke [2]. Since nNOS plays a critical role in the production of neuronal NO, it is considered to be a promising neuroprotective therapeutic target [36]. Inhibiting endothelial nitric oxide synthase (eNOS) and inducible nitric oxide synthase (iNOS) in this instance is undesirable, as these isoforms are responsible for maintaining crucial body function [32] and the inhibition of iNOS in certain regions, has been indicated to increase the probability of Alzheimer's disease [37]. Specific and selective inhibition of nNOS therefore provides a promising strategy in developing therapeutics for the treatment of neurodegenerative disorders. Typical experimental inhibitors of nNOS include 7-NI (**4**), L-NNA (**5**), L-NIO (**6**), TRIM (**7**) and ARL17477 (**8**) (IC_{50} values ranging from 0.1 – 8.3 μM, Figure 4), but their selectivity towards the neuronal isoform is still disputed [38–42]. Although intense research efforts have been devoted to the design and development of small molecules to selectively inhibit the activity of nNOS, none has been reported to enter clinical

trials for neurodegenerative disease. Several new classes of drug-like molecules for selective inhibition of nNOS have however been reported and patented in the last few years.

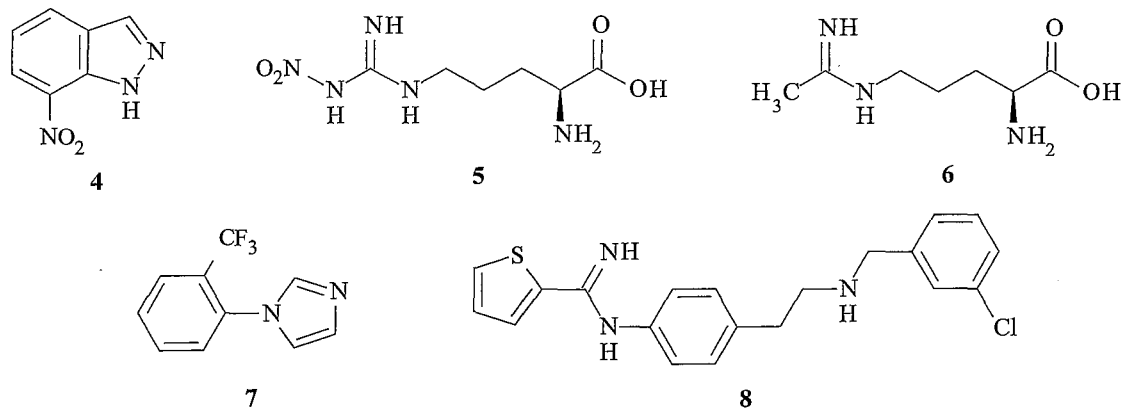


Figure 4: Typical structures of nNOS inhibitors described in literature

4.2.1.1. Neuraxon

Neuraxon patented several indole structures, represented by compounds **9** - **14** in Figure 5 [43-47]. These compounds were initially evaluated for NOS activity with the hemoglobin capture assay [48] using recombinant rat, bovine and murine NOS enzymes expressed in E. coli. Results from these screens indicated selectivity of the compounds towards nNOS inhibition. The indole derivatives were further evaluated on recombinant human iNOS, human eNOS and human nNOS produced in Baculovirus-infected Sf9 cells, using a radiometric method, measuring [^{3}H]*L*-citrulline production, and were reported to possess selective inhibition of nNOS with IC_{50} values ranging from 0.41 to 2.6 μM. Further studies by Neuraxon indicated that these derivatives were effective neuroprotective agents in various *in vitro* and *in vivo* neuroprotective studies. The compounds also showed ability as anti-hyperalgesics and anti-inflammatory agents, thereby indicating these indole nNOS selective inhibitors' potential as therapeutic agents against NO-derived neuropathic pain and in neurodegenerative disorders.

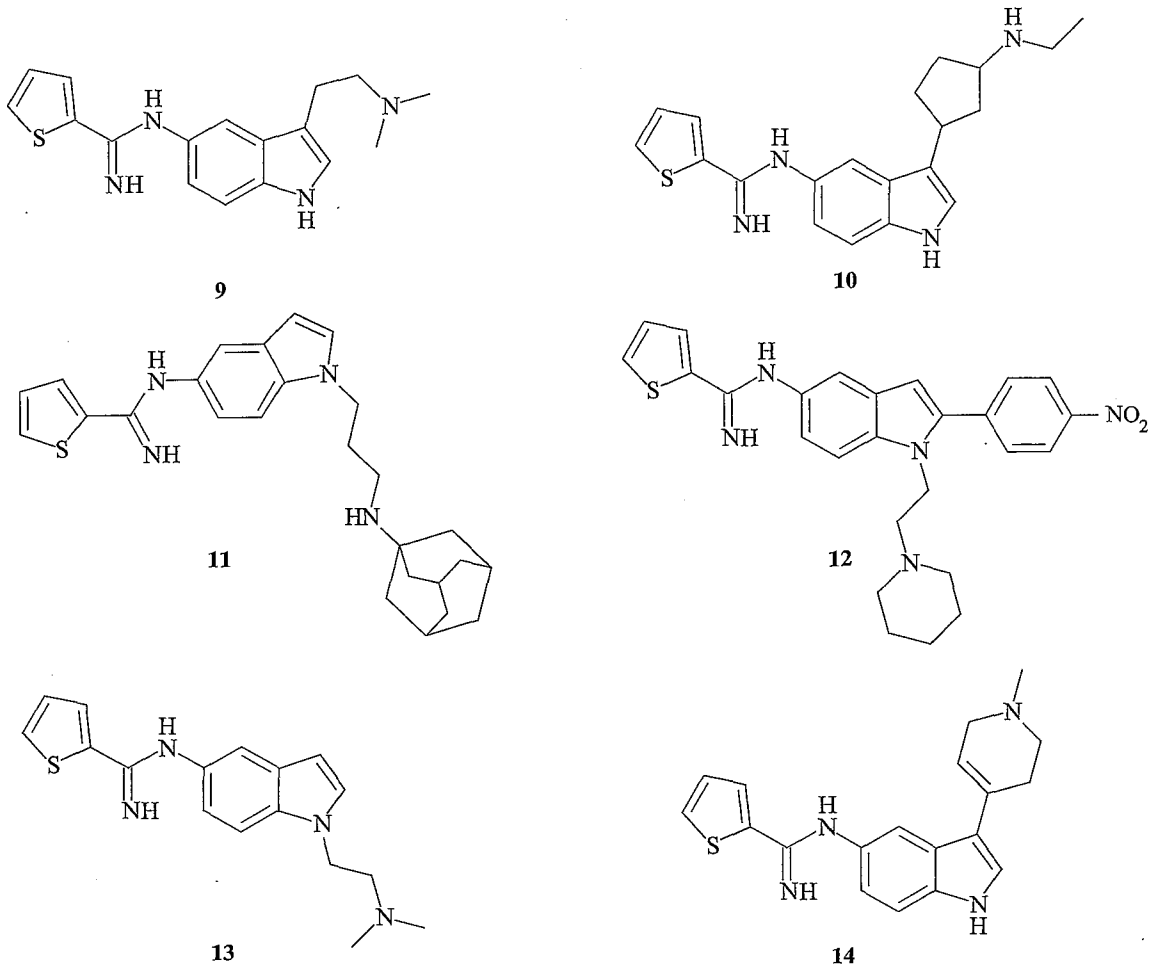


Figure 5: Neuraxon's indole derivatives as selective nNOS inhibitors

Other classes of molecules developed by Neuraxon as nNOS inhibitors (Figure 6) include quinolones, tetrahydroquinolones and related compounds (**15** - **17**), benzimidazoles (**18**) and the benzoxazines and benzothiazines (**19** and **20**) [49 - 51]. These derivatives possess selective inhibition of nNOS with IC_{50} values ranging between 0.01 and 0.44 μM. Compound **15** (IC_{50} = 0.01) was found to be 1420 and 290 times more selective for nNOS than for eNOS and iNOS, respectively. A common theme and probable pharmacophoric group in all of the Neuraxon structures is the thiophene-2-carboximidamide moiety conjugated to the aromatic ring of a further *N*-containing heterocyclic structure.

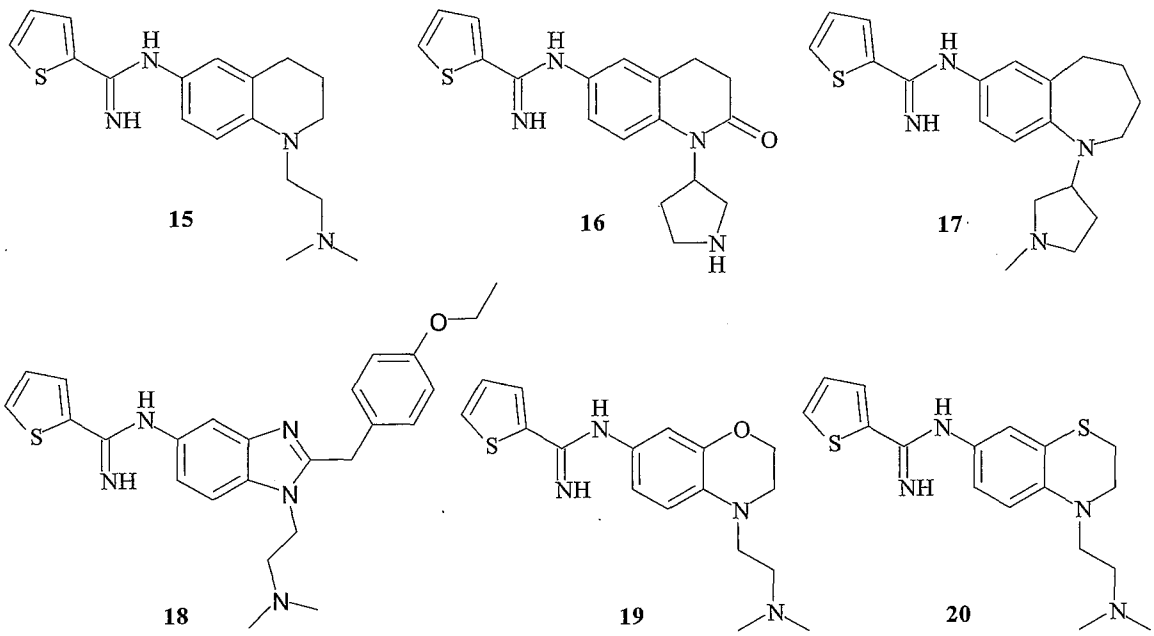


Figure 6: Additional structures patented as nNOS selective inhibitors by Neuraxon.

4.2.1.2. Northwestern University

Northwestern University has been highly active in the development of new nNOS inhibitors in the past 6 years. Through ongoing research of nNOS selective inhibitors, chiral-pyrrolidine based compounds **21** and **22** (Figure 7) were found to be highly potent inhibitors (K_i = 85 and 15 nM, respectively) [52-57]. The novel compounds were tested on recombinant enzymes of all 3 isoforms using the hemoglobin capture assay [48] and were found to be highly selective for nNOS over eNOS (1000- and 2100-fold, respectively) and iNOS (110- and 360-fold, respectively). However, results from animal studies indicated that these inhibitors did not optimally penetrate the blood-brain barrier (BBB), which impeded application thereof as candidates for treatment of neurodegenerative diseases. The decreased penetration of the BBB by passive diffusion was ascribed to the multiple nitrogen atoms on compounds **21** and **22** that were positively charged at physiological pH. Northwestern University addressed the permeability problems by designing new pyrrolidine based compounds (compounds **23** and **24**) with electron withdrawing groups, including monofluoromethylene and difluoromethylene, adjacent to the amine group in the lipophilic tail. These electronegative groups mitigated or partially removed the positive charge from the amine functionalities through electronegative induction, subsequently decreasing pKa values and improving membrane permeability [58, 59]. The compounds retained their potent selective nNOS inhibition (K_i = 36 nM, for both) over eNOS (1000- and 3800-fold, respectively) and iNOS

(360- and 1400-fold, respectively) and their monocationic character improved oral bioavailability.

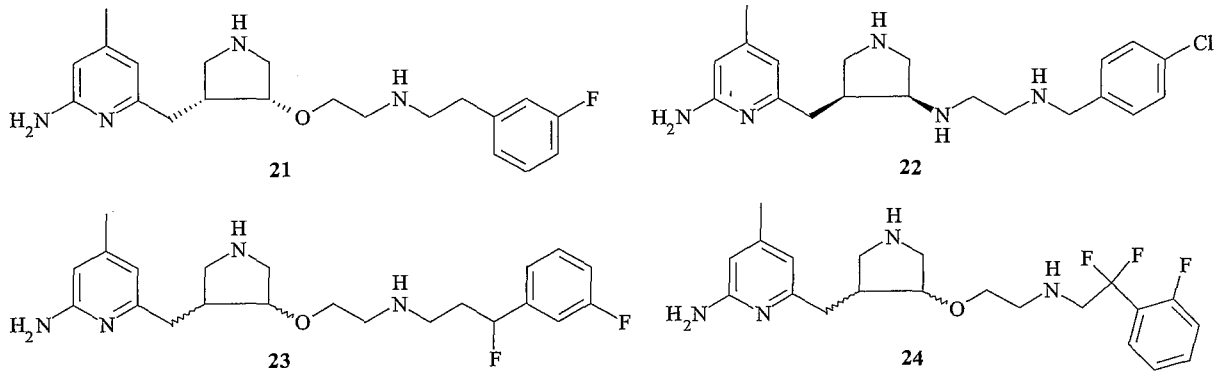


Figure 7: Pyrrolidine compounds as nNOS selective inhibitors

More recently, Northwestern University patented a series of aminopyridines (**25** and **26**, Figure 8) with potent nNOS inhibition (K_i = 28 and 103 nM, respectively) and selectivity over eNOS (96- and 156-fold, respectively) and iNOS (52- and 19-fold, respectively) [60]. They reported that the dimers have better oral availability and are more practical than the pyrrolidines for large scale synthesis and structure activity relationship studies.

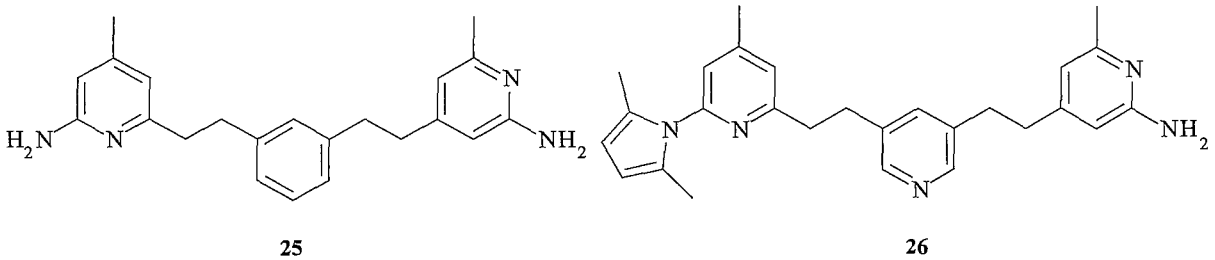
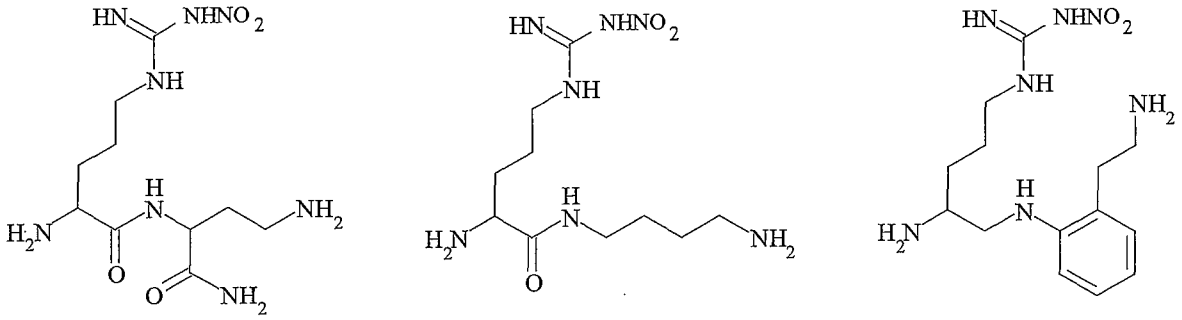


Figure 8: Aminopyridine compounds as nNOS selective inhibitors

A series of nitroarginine dipeptides (**27** – **29**) were also developed by this group [61-66] (Figure 9). These derivatives possess selective inhibition of nNOS with K_i values ranging between 0.01 μM and 0.44 μM. Compound **27** (K_i = 0.01 μM) was found to be 1420 and 290 times more selective for nNOS than eNOS and iNOS, respectively.



27 28 29

Figure 9: Nitroarginine dipeptide derivatives as nNOS selective inhibitors

4.2.2. Inducible Nitric Oxide Synthase Inhibitors

The iNOS enzyme is a homodimer composed of 130 kDa subunits. Each subunit comprises an oxygenase domain and a reductase domain. Importantly, dimerization of iNOS is required for enzyme activity. If the dimerization mechanism is disrupted, the production of NO *via* inducible NOS is inhibited [12]. The presence of iNOS in macrophages and lung epithelial cells is significant, and once present, iNOS synthesizes 100-1000 times more NO than the constitutive enzymes and does so for prolonged periods. This excessive production of NO and resulting NO-derived metabolites (e.g. peroxynitrite) elicit cellular toxicity and tissue damage, which contribute to the pathophysiology of a number of diseases, disorders and conditions [2, 11, 67]. iNOS-selective and nonselective inhibitors have been investigated for the treatment of iNOS-mediated diseases and conditions including pain, hypotension, inflammation, cerebral ischemia, arthritis, asthma and neuropathies such as diabetic neuropathy and post-hernia neuralgia [32, 68, 69].

Selective inhibition of inducible NOS seems to be a promising therapeutic approach for the treatment of the acute and chronic diseases mentioned above. Reports of selective iNOS inhibitors in animal models of acute and chronic diseases are however limited. In fact, only a few selective iNOS inhibitors are described and none has reached approved clinical application [70]. Earlier compounds tended to fall into the category of irreversible active-site inhibitors, leading to unacceptable side effects. As the important physiological roles played by the constitutive NOS isoforms, particularly eNOS, became more clear, later compounds were designed to have isoform selectivity to ensure that the iNOS inhibitors had the least possible effect on the activity of eNOS [71]. Data from selective iNOS inhibitors available, such as 1400W (**30**) [72], GW274150 (**31**) and GW273629 (**32**) [73, 74], AR-C102222 (**33**) [75, 76], ONO1714 (**34**) [77], *L*-arginine derivatives, L-NIL (**35**) and SC-51 (**36**) [78], and the

dimerization inhibitor BBS-1 (**37**) [79], show promising results in animal models of sepsis, lung inflammation, arthritis, and autoimmune diabetes (Figure 10).

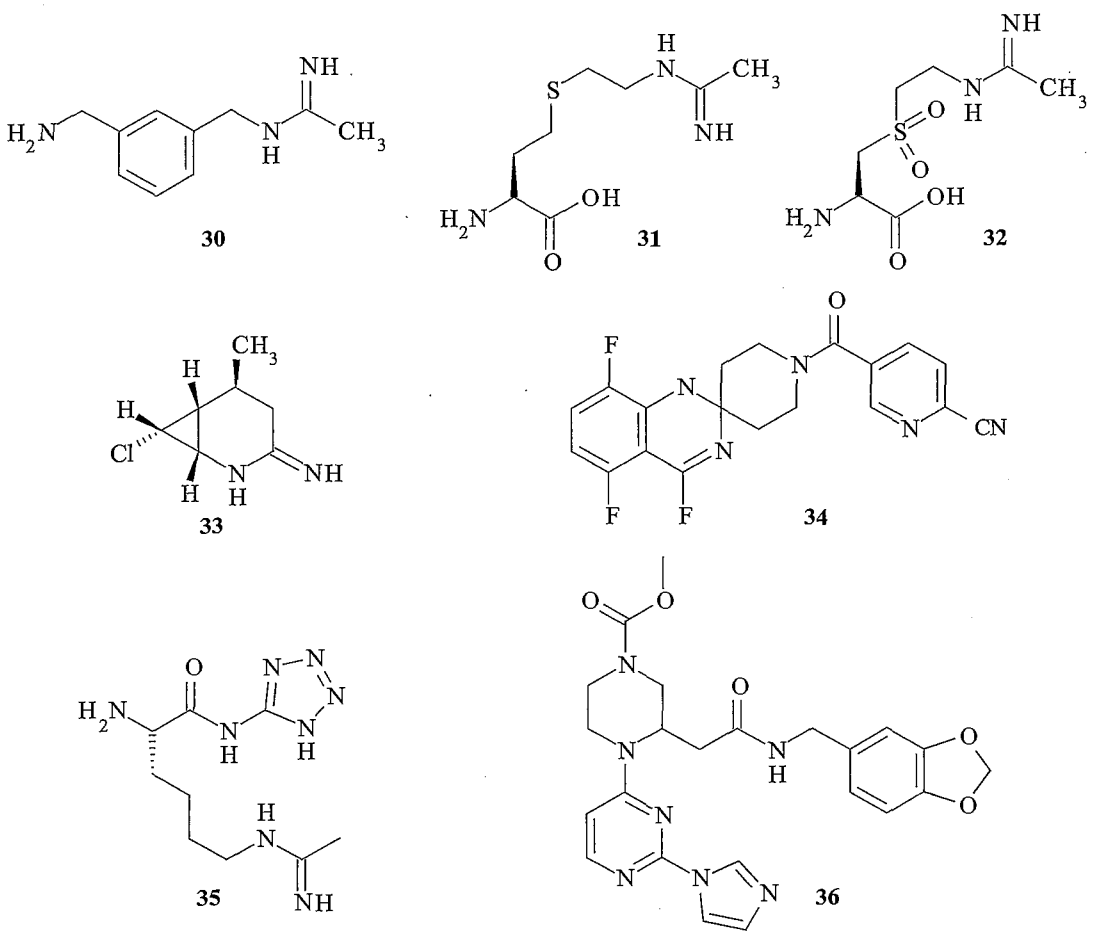


Figure 10: iNOS selective inhibitors with promising *in vivo* activity.

4.2.2.1. Altana Pharma

Altana Pharma patented two classes of selective iNOS inhibitors based on oxazolo[4,5-B]pyridines (**37** and **38**) and imidazole-substituted benzophenone scaffolds (**39** - **42**, Figure 11) [80-85]. These molecules were tested in human NOS isoform assays by means of radiometric measurement of the conversion of [^{3}H]arginine to [^{3}H]citrulline. The oxazolo[4,5-B]pyridine series were found to possess IC_{50} values for iNOS between 0.056 - 0.676 μM whereas, in the imidazole series, IC_{50} was measured at more than 0.32 μM . The two series of compounds were found to be highly selective for iNOS and inhibition of constitutive nitric oxide synthases (eNOS and nNOS) were measured only at a IC_{50} of 67.09 - 316.23 μM.

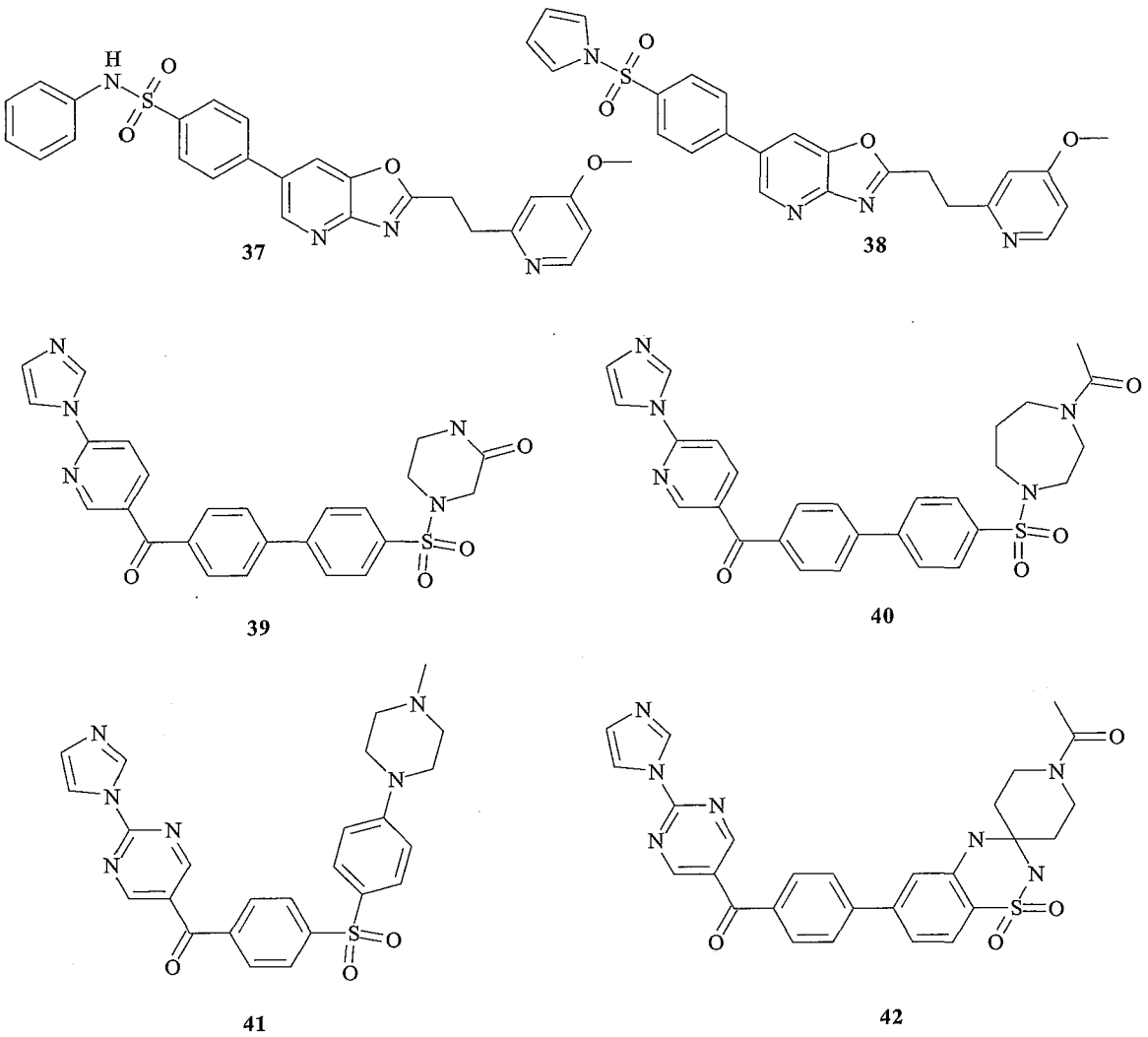


Figure 11: Oxazolo[4,5-B]pyridines (**37** and **38**) and imidazole-substituted benzophenones (**39 - 42**)

Altana Pharma also patented a series of imidazopyridine derivatives (**43** – **48**, Figure 12) with potent iNOS inhibitory activity (IC_{50} values ranging between 0.003 – 0.141 μM) [86-90]. Nothing is reported in literature on the selectivity of these derivatives.

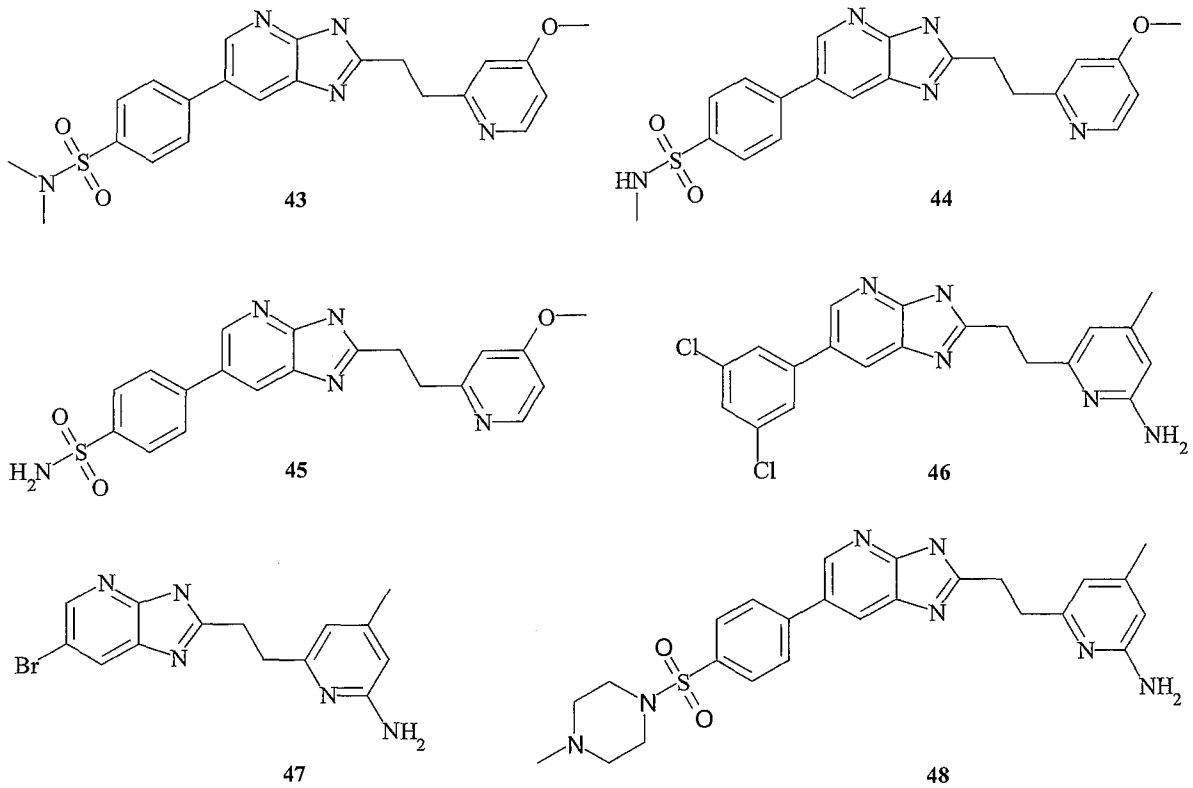


Figure 12: Imidazopyridine derivatives

4.2.2.2. Aventis Pharmaceuticals

Coumarin derivatives represented by **49** – **51** (Figure 13) were reported by Aventis to be potent selective inhibitors of both human iNOS (IC_{50} values of 0.094 – 0.51 μM) and mouse iNOS (IC_{50} values of 0.06 – 0.39 μM), with selectivity over the constitutive isoforms, especially eNOS (more than 100 fold) [91, 92]. The compounds were evaluated for NOS activity by radiometrically measuring the conversion of [^{3}H]-arginine to [^{3}H]-citrulline [93].

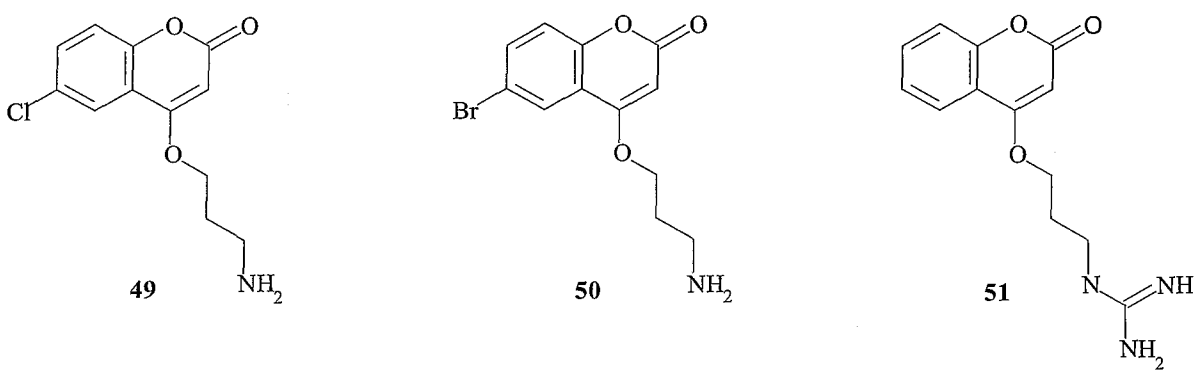


Figure 13: Coumarin based iNOS selective inhibitors

4.2.2.3. Nycomed

Imidazopyridine derivatives represented by **52** (IC_{50} = 0.2 μM) and **53** (IC_{50} = 0.170 μM) were patented by Nycomed to be highly effective inhibitors of iNOS (Figure 14). No results were reported on the selectivity of these compounds [94, 95].

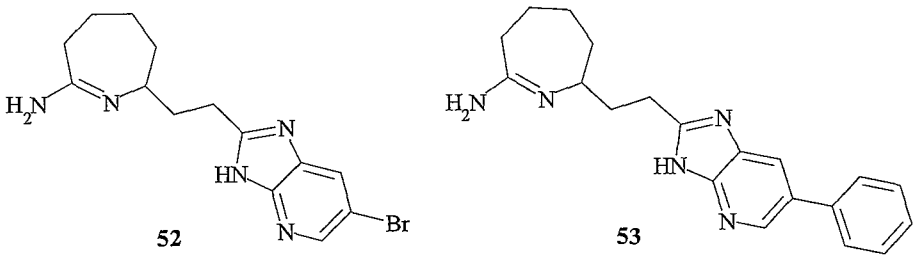


Figure 14: Recently patented iNOS inhibitor from Nycomed

4.2.2.4. Schering

N-Heterocyclic derivatives **54** - **57** (Figure 15) were patented by Schering as iNOS inhibitors [96, 97]. Fluorescent cell based NOS assays were used, employing the measurement of the NO oxidation product, nitrite, in RAW 264.7 murine macrophage and A172 cells. The efficacy of the compounds in treating adjuvant-induced arthritis in rats, resulting from an increase in nitric oxide production was determined by subcutaneous administration of the iNOS inhibitors. These compounds demonstrated the ability to treat adjuvant-induced arthritis by reducing nitric oxide overproduction.

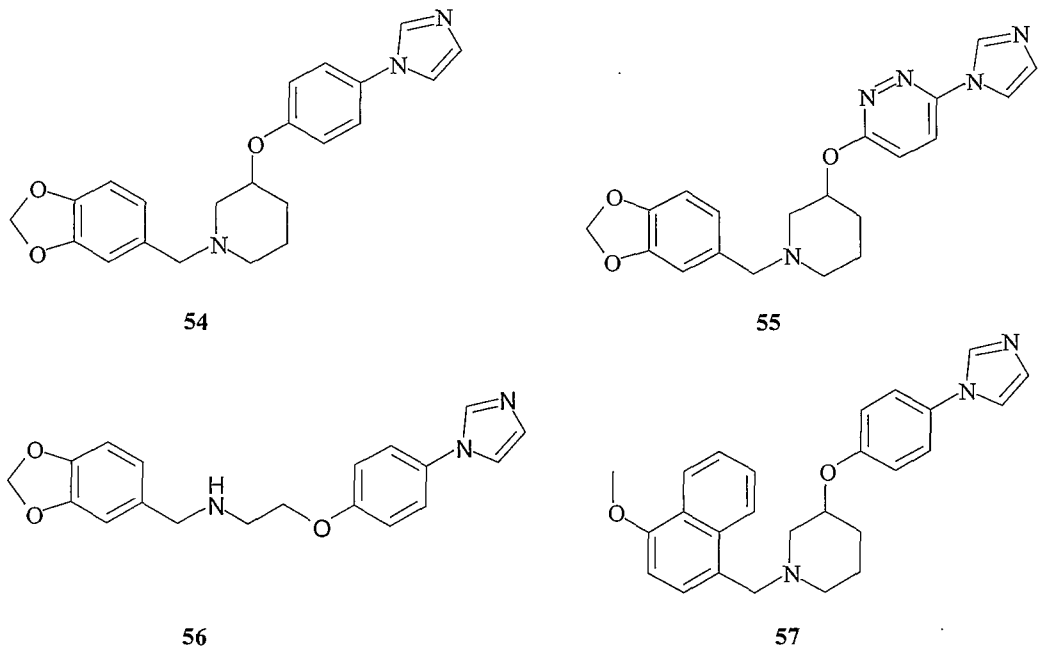


Figure 15: Schering's patented iNOS inhibitors

4.2.2.5. Berlex

Berlex described a series of heterocyclic derivatives **58** – **60** (Figure 16), structurally related to that of Schering, as iNOS inhibitors (IC_{50} < 25 μM) [98-100]. NOS activity was measured on the murine monocytic cell line, RAW 264.7, by a fluorescent assay of the nitrate, using 2,3-diaminonaphthalene as fluorescent nitrate detector [101]. These compounds also demonstrated the ability to treat adjuvant-induced arthritis present in rats by reducing nitric oxide overproduction.

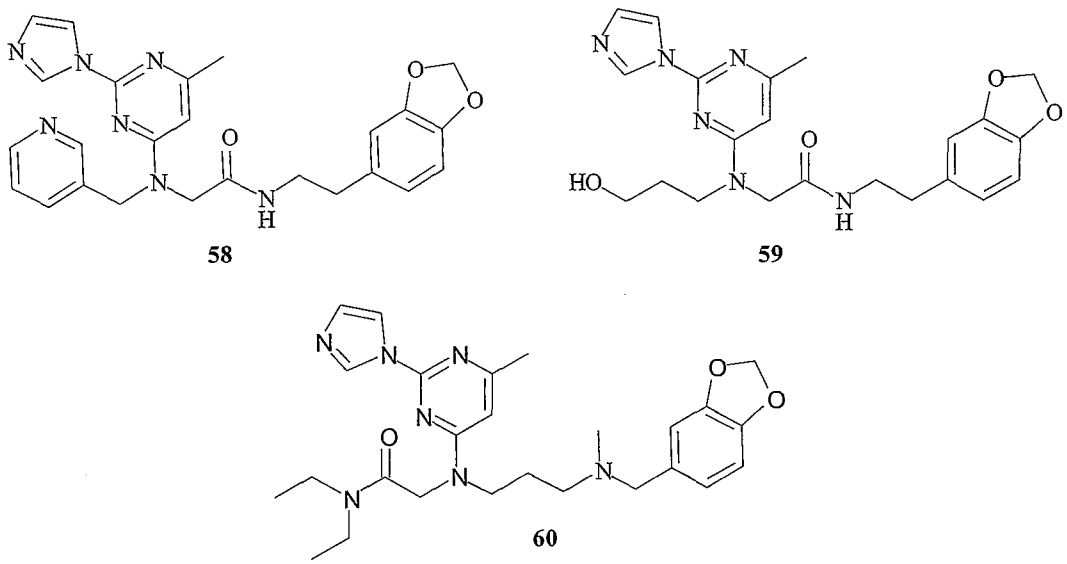
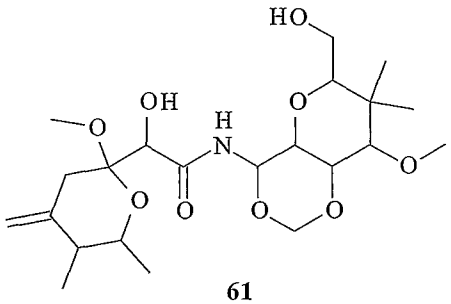


Figure 16: Berlex's compounds patented as iNOS inhibitors

4.2.2.6. Seoul National University Industry Foundation

Theopederin derivatives **61** and **62** were discovered by the Seoul national university industry foundation from the extract of *Porifera* (Figure 17) [102]. These compounds possess potent IC_{50} values of 0.0026 and 0.03 μM compared to the known iNOS inhibitor 1400W (**30**, IC_{50} = 0.01 μM) when tested for iNOS inhibition on RAW264.7 cells by measuring NO generation using the Griess reaction. These compounds were shown to have excellent iNOS inhibitory activity at very low concentrations without inducing cytotoxicity and could be effectively used as treatment agents for immune and metabolic diseases. No data was included on NOS isoform selectivity.



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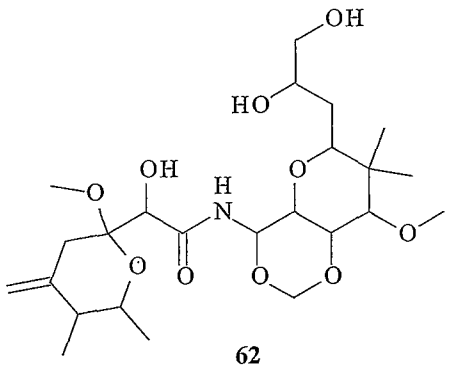

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Figure 17: Theopederin derivatives

4.2.2.7. Pharmacia Corporation

Pharmacia patented two classes of aliphatic amidino iNOS selective derivatives based on the rigid carbon-carbon double bond amino-alkyl (**63 – 66**) and the thio-alkyl (**67**) scaffolds (Figure 18) [103 – 107]. These molecules were tested *in vitro* on human NOS isoforms by radiometrically measured conversion of [^{3}H]arginine to [^{3}H]citrulline [93]. *In vivo* fluorescent based determinations were also performed on endotoxin-treated rats to induce systemic expression of iNOS, resulting in elevated plasma nitrite/nitrate levels [101]. Results obtained from these assays demonstrated iNOS selective inhibition by the derivatives *in vitro* (IC_{50} values of 0.36 – 1.4 μM) and *in vivo* (IC_{50} values of 0.1 – 0.4 μM) for both compound classes. The compounds were also found to be highly effective as iNOS inhibitors in the human cartilage explants assay, a model for osteoarthritis (IC_{50} values of 0.1 – 0.8 μM). The rigid carbon-carbon double bond amino-alkyl derivatives impart a favorable interaction with iNOS, such that the compounds have potency and selectivity for inhibition of iNOS over constitutive isoforms. The authors also surprisingly found that both series of compounds were less able to penetrate certain non-target organs in test systems, especially in comparison to related compounds of previous patents [108, 109]. This surprising differentiation in access between the target organ (cartilage) and other organs is an unexpected advantage of these molecules.

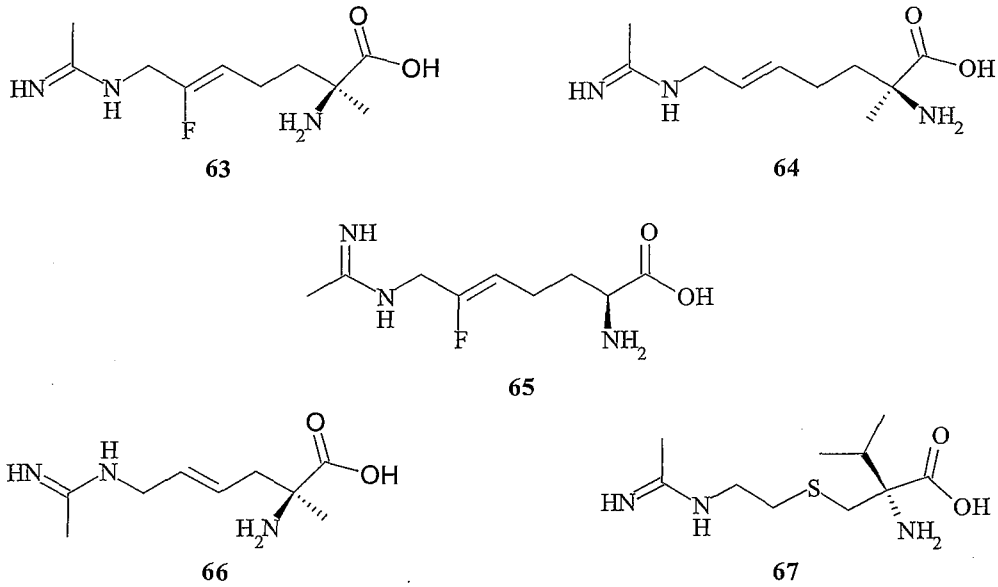


Figure 18: Pharmacia's amidino derivatives

4.2.2.8. Kalypsis

Kalypsis patented numerous series of compounds that exhibited inhibition of iNOS. Their compounds patented over the past 6 years were tested on HEK293 cells, transiently transfected with iNOS, eNOS or nNOS. The nitrite detecting fluorescent probe, 2,3-diaminonaphtalene (DAN), was used in rapid, quantitative fluorometric assays to quantify the activity of the compounds against the NOS isoforms [110]. Figure 19 represents a series of imidazole dimerization inhibitors (**68** - **73**) of the inducible NOS monomer developed by Kalypsis [111 - 115]. These derivatives exhibited inhibitory activity with IC_{50} values of less than 1 μM. These compounds were found to be iNOS dimerization inhibitors and no data on the selectivity thereof over the constitutive NOS isoforms was included. Further studies on the anti-inflammatory and analgesic activity of these compounds were conducted using various *in vivo* tests on rat models. The derivatives showed activity in these models and have potential as anti-inflammatory and analgesic compounds where there is an overproduction of NO derived from iNOS. Structural similarities between these structures and that patented by Berlex and Schering are clearly evident. All these compounds feature imidazole and benzodioxole moieties linked *via* carbon or heterocyclic structures. These seem to be important pharmacophoric groups for inhibition of iNOS dimerization.

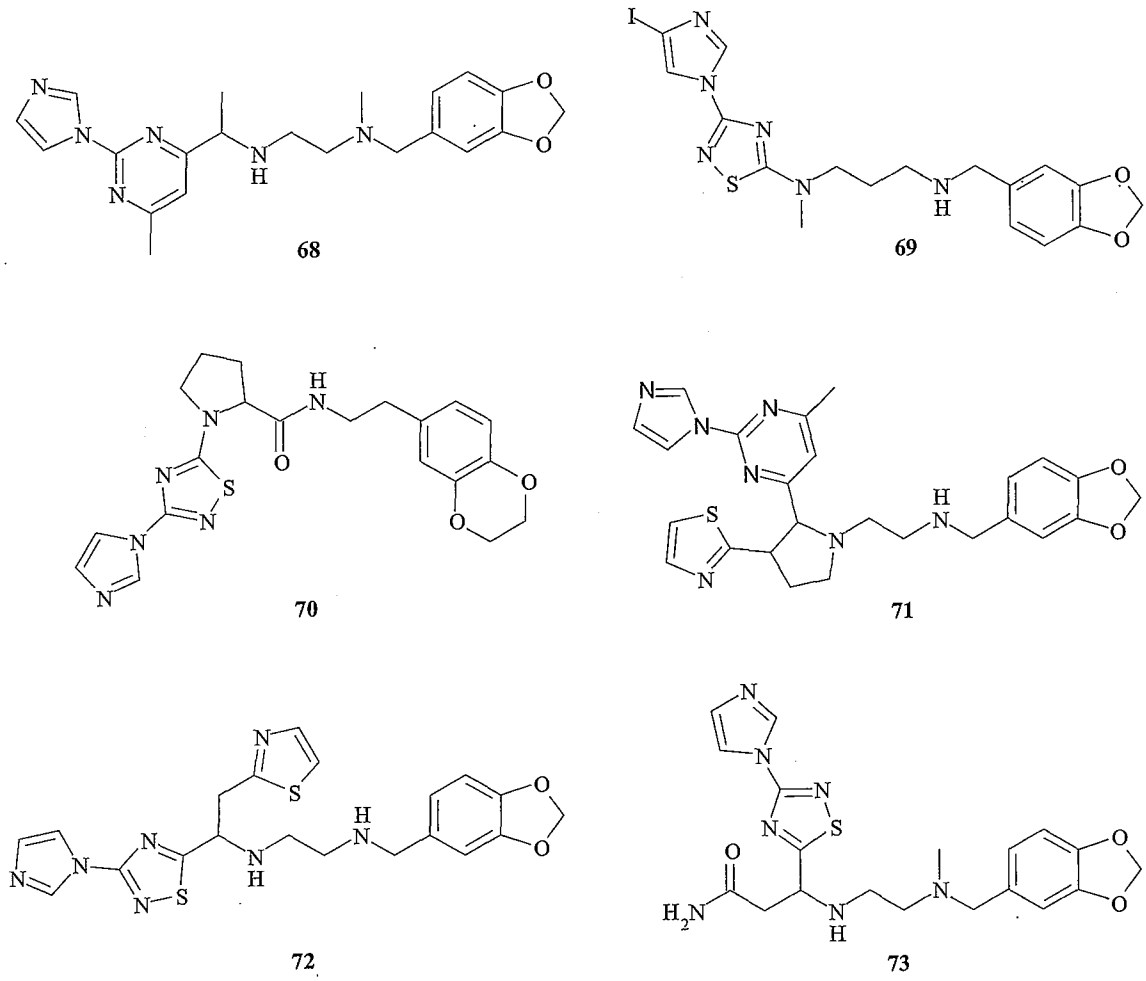


Figure 19: Imidazol derivatives as iNOS dimerization inhibitors

Kalypsis further filed several patents on a quinolone series represented by compounds **74** - **78** in figure 20 [116-121]. The quinolones were reported to possess inhibition of iNOS with IC_{50} values of less than 1 μM. The compounds were evaluated for their inhibition of induction of iNOS *via* the murine lipopolysaccharide (LPS) challenge. Injection of LPS was shown to induce iNOS transcription, increasing both iNOS and NO, leading to inflammation, edema, and the onset of sepsis [122]. Compounds **74** – **78** were found to inhibit LPS induced inflammation in rats to between 63% and 98% relative to a control at dosages below 30 mg/kg.

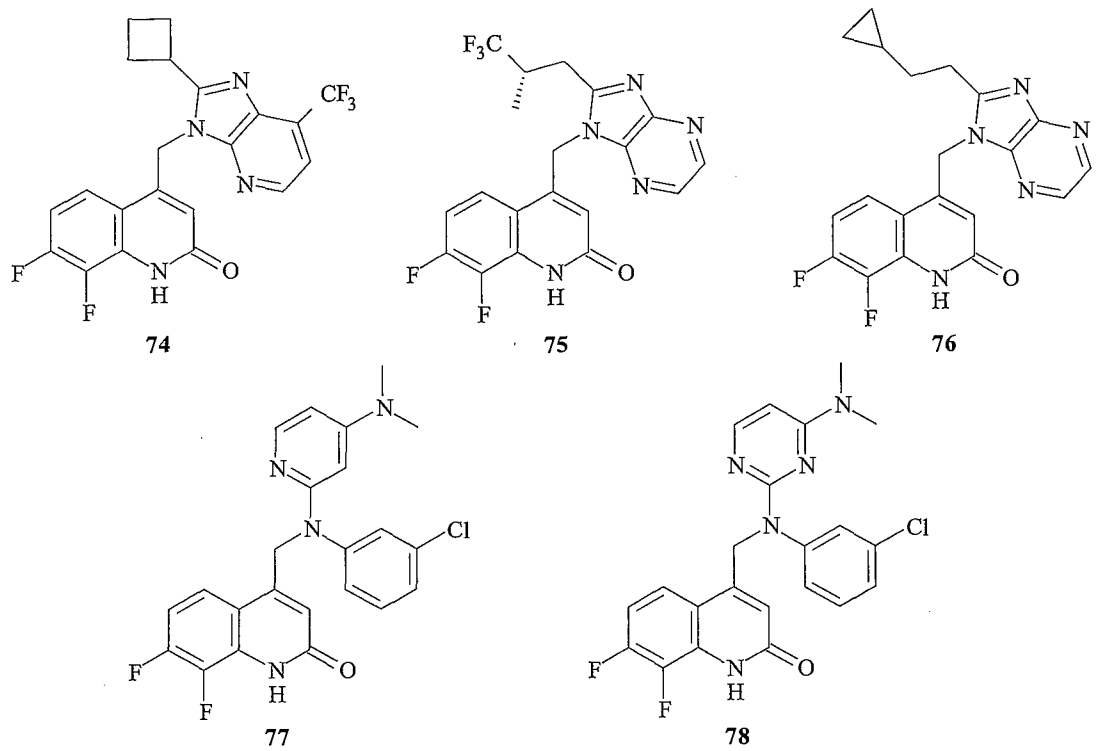


Figure 20: Kalypsis' quinolone based iNOS inhibitors

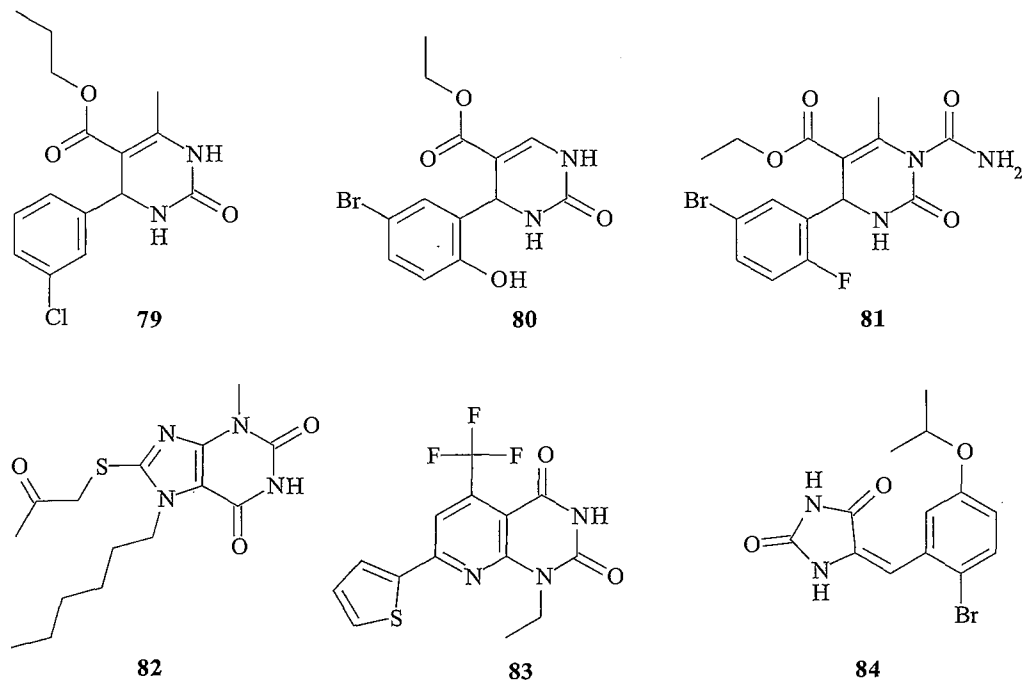
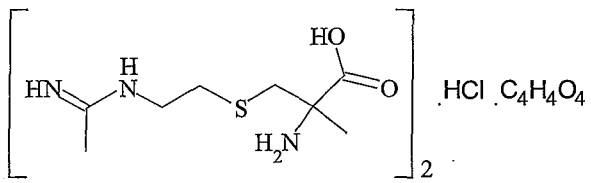


Figure 21: Other classes patented by Kalypsis

Other classes of molecules developed by Kalypsis as iNOS inhibitors that typically possess IC_{50} values of less than 5 μM included tetrahydropyrimidines (79 - 81), bicyclic pyrimidines (82, 83) and imidazolines (84), figure 21 [123, 124].

4.2.2.9. Pfizer

Pfizer recently developed a novel crystalline salt, *S*-[2[(1-iminoethyl)amino]ethyl]-2-methyl-*L*-cysteine maleate hydrochloride (**85**, Figure 22) [125]. The amorphous solid counterpart *S*-[2[(1-iminoethyl)amino]ethyl]-2-methyl-*L*-cysteine, was previously described and claimed in commonly assigned U.S. Pat. No; 6403830 [126]. Stoichiometrically, the novel crystalline salt contains two molecules of S-[2[(1-Iminoethyl)amino]ethyl]-2-methyl-*L*-cysteine with one molecule of maleic acid and one molecule of hydrochloride. It showed selective iNOS inhibition (IC_{50} = 3.1 μM) over eNOS and nNOS (IC_{50} = 77 and 15 μM, respectively) and may be used for the treatment of conditions involving an inappropriate expression of NO from the inducible isoform of NOS. Pfizer claimed that the compound would provide enhanced stability profiles and meaningful benefit over its less stable amorphous counterpart. This includes improved stability at ambient conditions and during the manufacturing processes. Limited polymorphic changes will also avoid the requirement of special storage conditions as well as frequent inventory replacement of compound **85**.



85

Figure 22: Pfizer's novel crystalline salt, *S*-[2[(1-Iminoethyl)amino]ethyl]-2-methyl-L-cysteine maleate hydrochloride

4.2.2.10. Astrazeneca

Astrazeneca patented a series of 3-arylthio-3-thiazolyl- (**86** and **87**), hetero- (**88**) and phenylarylamine (**89** - **91**) derivatives as NOS inhibitors with preference for the iNOS isoform (Figure 23) [127-132]. Inhibition of NOS by these compounds were determined by radiometrically measuring the formation of *L*-[^{3}H]citrulline from *L*-[^{3}H]arginine using an adaptation of the method by Fostermann *et al* [133]. The compounds were screened for NOS inhibition using the enzyme as prepared from the cultured murine macrophage cell line J774A-1. Compounds **86** – **91** had IC_{50} values of less than 10 μM against the crude NOS enzyme, indicating that they are expected to show useful therapeutic activity. The compounds were further evaluated against recombinant human NOS (iNOS, eNOS or nNOS) expressed in *E.coli* using the adapted method of Fostermann *et al.* In this evaluation compounds **86** – **88**

exhibited IC_{50} values of less than 10 μM for iNOS. No data was included on the selectivity of these compounds against the human constitutive isoforms of NOS. In the same assay compounds **89 – 91** showed highly potent inhibition of both iNOS (IC_{50} values ranging from 0.0029 - 0.16 μM) and nNOS (IC_{50} values ranging from 0.0048 - 0.2 μM) with good selectivity with respect to the inhibition of eNOS (IC_{50} values ranging from 0.34 – 44 μM). Compounds **86 – 91** also showed NOS inhibitory activity in the human colorectal carcinoma cell line, DLD-1 where Griess reagent was used to determine the nitrite levels produced by cells treated with the iNOS inhibitors. The IC_{50} values of the test compounds were measured at less than 100 μM, indicating that these compounds should show useful therapeutic activity for the treatment of conditions involving an inappropriate expression of nitric oxide from iNOS.

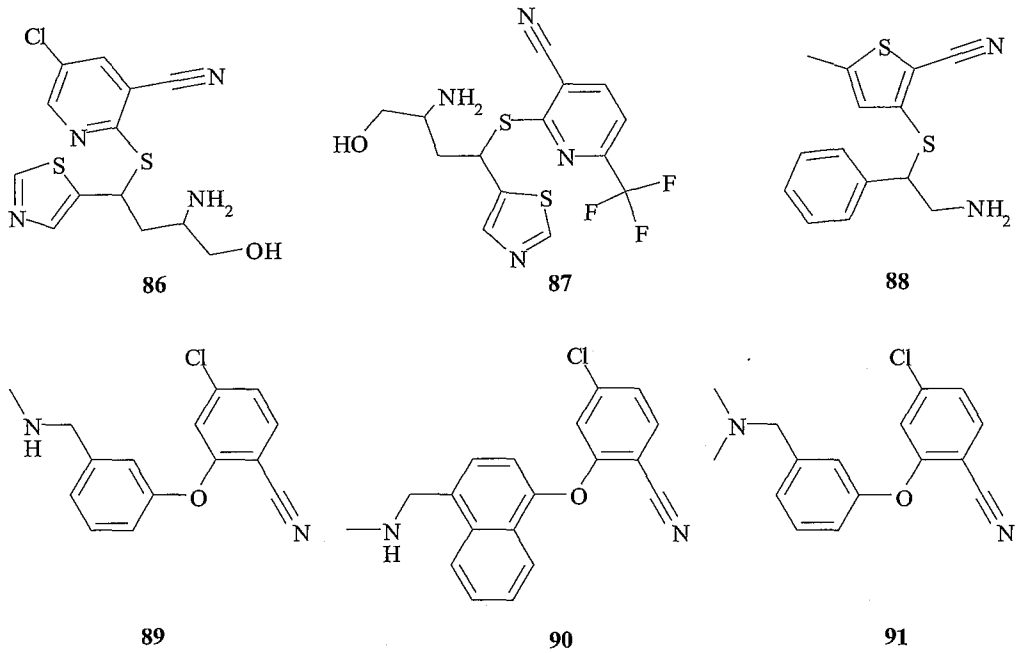


Figure 23: Arylamine derivatives patented by AstraZenica

4.2.3. Endothelial Nitric Oxide Synthase Inhibitors

NO, produced by eNOS, is a key signaling molecule in vascular homeostasis including vascular tone and blood pressure [134]. In addition, NO has multiple antiatherogenic roles, including anti-inflammatory, antithrombotic and antiproliferative effects [134]. A decreased concentration of NO is a cardinal feature of the endothelial dysfunction [135] that precedes the development of overt atherosclerosis and is an independent predictor of adverse cardiovascular risk [136]. Several factors contribute to loss of NO in endothelial dysfunction

states, including both reduced NO synthesis and NO scavenging by reactive oxygen species [137]. Inhibition of eNOS function would thus lead to unwanted effects such as enhanced white cell and platelet activation, hypertension and increased atherogenesis [32]. It is therefore imperative that therapeutic inhibitors of NOS do not affect eNOS as this will lead to unwanted side effects. Currently there are no patents on drugs that selectively inhibit eNOS and inventors are rather trying to develop novel inhibitors of NOS that does not affect eNOS while inhibiting nNOS and/or iNOS.

4.3. Expert Opinion

Although NO mediates several physiological functions, a number of disease states are associated with the overproduction thereof, or overstimulation by NO. This makes the NOS pathway an attractive target for the development of therapeutics, especially where immune function and/or inflammation forms part of the disease aetiology. NO produced by eNOS has however mainly a physiological role and the inhibition thereof leads to unwanted effects. The inhibition of nNOS or iNOS, but not of eNOS, could therefore provide effective therapeutic approaches and selective inhibitors could also be useful tools for investigating the biological functions of NO and the role thereof in disease. Since the isoforms possess a distinct cellular localization and are differently regulated, they can be specifically targeted for potential therapeutic approaches.

iNOS inhibitors have been under intense scrutiny as potential drugs for numerous immune and anti-inflamatory diseases and looking at the number of patents published describing the development of iNOS selective drugs, the possible value in pursuing the development of an iNOS inhibitor for therapeutic use is definitely recognised. As an extension of this approach, an iNOS-NSAID drug for the treatment of inflammation and pain also seems to be a viable option.

In the treatment/prevention of neurodegenerative disorders, nNOS inhibition alone may not be an effective treatment option because of the multitude of mechanisms that may lead to neurodegeneration. The compounds described by Neuraxon for example are indicated to be not only nNOS selective inhibitors, but also *N*-methyl-D-aspartic acid receptor antagonists, thus affecting more than one pathway to attenuate the progression of neurodegeneration. The ideal drug in this case would be a dual targeted or multifunctional drug [138], with selective inhibition of nNOS and limited or no effect on iNOS and eNOS.

Over the past 6 years, more than 60 new patents on compounds with activity as NOS inhibitors were filed. NOS inhibitors have received considerable interest and much research effort and funding were spent by pharmaceutical companies and academic institutions in the development of potentially novel therapeutic strategies utilizing this mode of action. In literature, and also in the reviewed patent literature, the progression from the classical transition state analogues (*L*-NAME, **1**, *L*-NMMA, **2**, and aminoguanidine, **3**) towards significantly more bulky *N*-containing heterocycles, mostly still incorporating guanidine-like or amidino moieties, or bioisosters thereof, with both basic and acidic nitrogen atoms is clear. From the patent literature it is also evident that extended conformations with multiple hydrophobic/aromatic and hydrogen bond interaction sites – often including imidazole structures - are favored. Novel structures isolated from nature, like the theopederin derivatives (**61** and **62**) of Seoul National University Industry Foundation, are the exception to this, exhibiting potent iNOS inhibitory activity for the predominantly *O*-containing non-aromatic polycyclic structures.

Recent structures in the current literature mostly result from high-throughput screening but the solved crystal structures of the NOS isoforms could prove pivotal in future structure-based drug design for these enzymes. The challenge herein lies in the fact that from the crystal structures of the catalytic domains of all three NOS isoforms, the active sites are nearly identical, making structure-based design of isoform–selective inhibitors a difficult and challenging problem. Northwestern University's compounds are an example of what can be achieved by using structure-based design to develop novel potent and selective NOS inhibitors by crystallographic analysis and computer modeling [52-57]. Using this approach, together with what they learned from previous studies [61-66, 139] the pyrrolidino compounds (**21**, **23**, **24**) were designed and synthesized, and exhibited nanomolar nNOS inhibitor potency and more than 1000-fold nNOS selectivity over eNOS. These crystal structures and advances in molecular modeling will, without doubt, pave the way for the discovery of novel, potent and highly selective inhibitors of the NOS isoforms.

Despite major advances over the past few years, the present state of investigation of NOS inhibitors is not without flaws. One relates to the almost exclusive reliance on rat or mouse models for *in vivo* efficacy studies, which may not directly correlate to efficacy in humans. Since experiments in non-human primates are expensive and becoming ethically questionable, alternative animal models will need to be validated for more accurate NOS inhibitor candidate

assessment, to firmly establish the mechanism of action and therapeutic potential of some of these compounds. Further success in the discovery of NOS inhibitors will also clearly depend on a better understanding of NO at the molecular, cellular and tissue levels. Many of the newly identified NOS inhibitors described here may never progress beyond the experimental stage due to insufficient efficacy, selectivity or prohibitive toxicity in animal studies. The bulk of the promising agents obtained to date also did not have pharmacokinetic properties suitable for orally-administered agents that would have good patient compliance. As a result the main challenges in the development of therapeutically acceptable NOS inhibitors remain - to design a potent, selective inhibitor of specific NOS isoforms with high oral bioavailability and a low side effect profile. In this instance pharmacodynamic, temporal and regional selectivity could be an requirement.

The NOS isoforms however, remain attractive targets for combating various pathological conditions. Developing techniques like *in vivo* imaging fluorescent molecular probes for these enzymes could also provide valuable information in disease progression. This could also lead to more information on pharmacodynamic questions like the molecular and structural basis of high isoform selectivity of compounds. If research on NOS enzymes continues for another decade at anything like the current level, it will surely produce answers for many of the existing questions, and may well result in the development of novel drug therapies. Exciting times are ahead in the search for novel NOS inhibitors, both in terms of basic pharmacology and clinical drugs.

Declaration of interest

The authors state no conflict of interest and have received no payment in preparation of this manuscript.

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