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# Inducible Nitric Oxide Synthase: Regulation, Structure, and Inhibition

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## Abstract

A considerable number of human diseases have an inflammatory component, and a key mediator of immune activation and inflammation is inducible nitric oxide synthase (iNOS), which produces nitric oxide (NO) from L-arginine. Overexpressed or dysregulated iNOS has been implicated in numerous pathologies including sepsis, cancer, neurodegeneration, and various types of pain. Extensive knowledge has been accumulated about the roles iNOS plays in different tissues and organs. Additionally, X-ray crystal and cryo-EM structures have shed new insights on the structure and regulation of this enzyme. Many potent iNOS inhibitors with high selectivity over related NOS isoforms, neuronal NOS (nNOS) and endothelial NOS (eNOS), have been discovered, and these drugs have shown promise in animal models of endotoxemia, inflammatory and neuropathic pain, arthritis, and other disorders. A major issue in iNOS inhibitor development is that promising results in animal studies have not translated to humans; there are no iNOS inhibitors approved for human use. In addition to assay limitations, both the dual modalities of iNOS and NO in disease states (i.e., protective vs. harmful effects) and the different roles and localizations of NOS isoforms, create challenges for therapeutic intervention. This review summarizes the structure, function, and regulation of iNOS, with focus on the development of iNOS inhibitors (historical and recent). A better understanding of iNOS' complex functions is necessary before specific drug candidates can be identified for classical indications such as sepsis, heart failure, and pain; however, newer promising indications for iNOS inhibition, such as depression, neurodegenerative disorders, and epilepsy, have been discovered.

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## 1. INTRODUCTION

Nitric oxide (NO) is an important cellular signaling molecule that participates in diverse physiological functions in mammals, including vasodilation, smooth muscle relaxation, neurotransmission, and the immune response. NO, a free radical, is produced by a family of enzymes called nitric oxide synthases (NOSs) by the oxidation of L-arginine (L-Arg) to L-citrulline. There are three isoforms of NOS. Two of them, neuronal NOS (nNOS) and endothelial NOS (eNOS), are constitutively expressed, while the third one is inducible and is thus termed iNOS. nNOS is primarily found in the nervous system and is necessary for neuronal signaling, while eNOS is localized to the endothelium and is essential for vasodilation and control of blood pressure.[1] These two isoforms produce nanomolar amounts of NO for short periods of time (seconds to minutes) in a calcium/calmodulin (CaM)-dependent manner.[2,3] iNOS, by contrast, is not constantly present in cells and is only expressed when the cell is induced or stimulated, typically by pro-inflammatory cytokines and/or bacterial lipopolysaccharide (LPS).[3,4] Upon induction, iNOS generates significant amounts of NO (micromolar range), which lasts until the enzyme is degraded, sometimes for hours.[5] The considerable amount of NO produced helps to defend against invading pathogens and is thus critical for the inflammatory response and the innate immune system. Inappropriately high NO concentrations from overexpression or dysregulation of iNOS, on the other hand, can result in toxic effects and is associated with a variety of human diseases, including septic shock, cardiac dysfunction, pain, diabetes, and cancers.[4] The dual activity of iNOS-related NO (beneficial vs. detrimental) is highly concentration-dependent. Therefore, regulation of its production is important for both maintaining its proper physiological functions and controlling its deleterious effects. Because of its important role, iNOS research is a very popular field; a SciFinder search for “iNOS” reveals over 37,000 references. In this contribution, we present a general review of iNOS, with a focus on its structure, functions, and regulation, both physiologically and pathologically. Inhibition of iNOS as a potential therapeutic strategy and iNOS inhibition in clinical trials also will be discussed.[6]

## 2. STRUCTURE AND FUNCTIONS OF iNOS

iNOS is a 131 kDa mammalian protein composed of 1,153 amino acids, which are assembled into two major domains, a C-terminal reductase—containing a flavin mononucleotide (FMN) binding subdomain—and a N-terminal oxygenase.[7] iNOS adopts a zinc-bridged, homodimeric quaternary structure that allows the enzyme to convert L-Arg to L-citrulline with the concomitant production of NO. This transformation is governed by an elaborate electron transport chain, involving the cofactors nicotinamide adenine dinucleotide phosphate (NADPH), flavin adenine dinucleotide (FAD), flavin mononucleotide (FMN),

heme, and (6*R*)-5,6,7,8-tetrahydrobiopterin ($H_4B$), which are essential for this reaction. This process is mediated by CaM, which binds in a hinge region between the oxygenase and reductase domains.[8] While iNOS, nNOS, and eNOS share substantial gene alignment, cofactors, and overall function, differences in domain architecture allow for specialization of function and regulation. iNOS is the simplest mammalian NOS in terms of domain content, consisting solely of the reductase, oxygenase, and CaM-binding domains; it lacks the auto-inhibitory loop present in eNOS, as well as the PDZ domain of nNOS.[9]

All NOS enzymes have a catalytically inactive zinc at the dimer interface.[10] One zinc atom per dimeric pair forms a zinc tetrathiolate cluster with each monomer donating two cysteine residues in a -CXXXXC- amino acid arrangement.[11] Biochemical investigation has determined that the role of the zinc ion is to promote $H_4B$ binding.[12]

Although CaM is associated with all NOS isoforms, the tight binding of CaM to iNOS (because of sequence variation at the hinge region)[13] allows it to activate at much lower physiological concentrations of calcium (40 nM in iNOS vs. 400 nM in nNOS and eNOS).[14] Given that typical cytoplasmic calcium concentrations are near 100 nM,[15] iNOS is effectively locked in an active position where regulation by calcium is no longer relevant. Post-translational modifications may also have functional or regulatory significance. For example, phosphotyrosine residues have also been detected in iNOS, and assays have shown that inhibition of cellular phosphatase activity by vanadate leads to increased iNOS activity.[16]

### 2.1 NO production mechanism

Although the driving force behind the production of NO is NADPH, the catalytic cycle proceeds through an elaborate intra- and inter-protein electron transport chain. The cycle begins when NADPH binds in the reductase domain and initiates the thermodynamically favored reduction (40 mV) of the adjacent FAD in a two-electron process (Fig. 1). Within the reductase domain, FAD is proximal to the FMN subdomain, which allows a one-electron reduction of FMN by FAD (6 mV).[17] At this point in the cycle, the spatial transfer of electrons from one domain to another is unclear, as the FMN subdomain is distal to the heme-containing oxygenase domain. Studies have shown a great deal of flexibility within the protein and have elucidated the conformational changes during the catalytic cycle.[18] Upon CaM and calcium sensitization, the enzyme contorts the FMN subdomain into proximity with the heme-containing oxygenase domain. Notably, the FMN domain does not transfer electrons to heme in its own monomer but rather into the dimeric protein's other monomer.[19] This explains why monomeric iNOS, and NOS enzymes in general, are inactive. Electrons received from FMN affect the reduction of heme iron from iron(III) to iron(II) and prime the active site by allowing the recruitment and activation of molecular oxygen. The exact mechanism of this activation is subject to debate.[20] However, near the active site there is a binding region for $H_4B$, which is believed to play a number of roles in the production of NO. $H_4B$ causes a high spin iron configuration in heme,[21] stabilizes the dimeric form of the enzyme,[22] and it may promote efficient NADPH coupling to NO production.[23] Furthermore, during the oxidation of L-Arg it is proposed to play a vital role as an electron shuttle and reservoir within the active site.[24] When L-Arg binds to an active

site L-Glu residue, the heme cofactor executes a P450-like mechanism to oxidize L-Arg to $N^{\omega}$-hydroxy-L-arginine (L-NOHA). A second distinct step then further oxidizes L-NOHA to L-citrulline and NO. At two points in the transformation of L-Arg to NO, $H_4B$ provides stabilization of the charge during the activation of molecular oxygen.[2] Donation of an electron from $H_4B$ to a heme iron(IV) hydroperoxyl species allows formation of the proposed catalytically active iron(IV) oxenoid radical cation.[25] In the final step, which releases NO from heme, $H_4B$ recaptures an electron from iron, thereby polarizing the iron-nitrogen bond and allowing NO to diffuse out of the active site.[26] During catalysis, $H_4B$ donates two electrons but recaptures only one. The source of the other electron is the FMN subdomain;[27] however, the exact mechanism and spatial organization required to pass this electron remain unknown.

### 2.2 Spatial organization of iNOS

A wealth of earlier biochemical evidence suggests that electrons are passed from the FMN subdomain into the heme in the oxygenase domain of the other monomer. However, until recently, there was no answer to fairly simple questions regarding the spatial organization of the domains and their movements to achieve catalysis. This problem largely stems from the inability to crystallize full-length dimeric NOS enzymes. In fact, crystal structures of the three isoforms' dimeric oxygenase domains were only solved beginning in the late 1990s and completed in 2002 with the publication of the nNOS structure.[11,28,29] Unlike nNOS or eNOS, the tight interaction between CaM and iNOS allowed their co-crystallization. This cemented the localization of CaM in NOS enzymes, but still left the overall organization unclear. In 2014, Marletta *et al.* published a cryo-EM study of the NOS enzymes that showed the three-dimensional domain architecture of the NOS family (Fig. 2) and visualized snapshots of the catalytic cycle in action.[18] They found that iNOS folds almost in half to deliver an electron from FMN to heme. While the resolution of such images is limited to domain structure, computational fits of the observed three-dimensional shape have suggested a high degree of flexibility in the CaM binding region, as well the ability of the FMN subdomain to move long distances independently of the reductase domain it canonically resides in.

### 2.3 Critical residues in iNOS

Mutational analysis of iNOS has identified a collection of amino acids that are critical for the function of the enzyme. When mutated to asparagine, Glu546 lowers the measured inter-domain electron transfer between the FMN subdomain and heme in the oxygenase domain. The rate constant was decreased by a third through a decrease in inter-domain binding affinity, as confirmed by electron paramagnetic resonance (EPR).[19] In the $H_4B$ binding pocket in the oxygenase domain, residues Arg375, Trp455, Trp457, and Phe470 are crucial for $H_4B$ cofactor binding and subsequent dimerization and function of the enzyme.[30] Arg375 is also implicated in modulation of the redox function of $H_4B$.[31] Phe831 and Leu832 in the reductase domain are integral to the hydrophobic core. When they are exchanged for polar residues—serine and proline, respectively—large decreases in iNOS activity result.[32] Unsurprisingly, the mutation of heme-ligating Cys194 to alanine results in failure to bind heme.[30] However, in addition to lacking heme, the enzyme fails to dimerize. The interaction of heme propionate and $H_4B$ is well known,[32] and thus it is possible that

heme-lacking monomers also lack $H_4B$, which is crucial for the dimerization process.[33] CaM binding is destabilized by the mutation of Ser562 and Cys563 in iNOS through the destruction of a crucial hydrogen bond network between CaM and iNOS.[34]

### 2.4 Biological function of NO produced by iNOS

The primary role of iNOS in human physiology is the destruction of invading pathogens.[35] As discussed previously, iNOS and NO are produced as a part of the immune response and have a regulatory role. As NO is readily diffusible, it can have a variety of fates. NO's high affinity for iron allows it to break up or inactivate heme-containing enzymes or iron-sulfur clusters.[10] NO can be responsible for cysteine nitrosylation[36] or can combine with superoxide to form $ONOO^-$ (peroxynitrite), a potent oxidant and nitrating agent. NO and $ONOO^-$ can cause DNA damage via oxidative deamination.[37] The short biological half-lives of NO and $ONOO^-$ direct these damaging mechanisms preferentially to the invading pathogen, thus avoiding systemic toxicity. Finally, cyclic guanosine monophosphate (cGMP) production is increased through NO mediated activation of guanylate cyclase. cGMP is a soluble second messenger that participates in many downstream signaling cascades through activation of various protein kinases, ion channels, and phosphodiesterases.[39–41]

## 3. REGULATION OF iNOS ACTIVITY

The activity of expressed iNOS is regulated by different factors, including availability of its substrate and cofactors, interaction with other proteins, and auto-inactivation.

### 3.1 Substrate and cofactor availability

L-Arginine, the substrate for iNOS, is also essential for biosynthesis of proteins and other amino acids and removes toxic ammonia from the body during the urea cycle. In the urea cycle, arginase converts arginine to ornithine and urea, and this affects iNOS activity by competing for the same substrate (Fig. 3). Indeed, arginase inhibition has been found to increase NO synthesis in rabbit and rat macrophages[42] and increases the amount of nitrated and S-nitrosylated protein in inflamed mouse lungs, possibly by enhancing NO production.[43] On the other hand, arginase overexpression can inhibit iNOS activity and promote dysregulated inflammatory responses. For instance, arginase overexpression in human keratinocytes is implicated in the disease mechanism of psoriasis, an inflammatory skin disease, possibly as a result of decreased iNOS activity.[44]

The availability of L-Arg is also controlled by the cation amino acid transporters, CAT1, CAT2 and CAT3,[45] that transport it (Fig. 3). Conditions used to induce iNOS also help to increase expression of arginine transporters, thus increasing arginine uptake. In rodent macrophages, induction of iNOS by LPS is accompanied by an increase in CATs and increased L-Arg uptake.[46] Likewise, deficiency in these transporters reduces iNOS activity. Deletion of the CAT2 arginine transporter gene impairs iNOS activity in stimulated astrocytes by 84% and also reduces NO synthesis in macrophages.[47]

Additionally, L-citrulline, the by-product of NO production, can be converted back to arginine through arginosuccinate synthase (Fig. 3). iNOS and arginosuccinate synthase are co-induced in macrophages, vascular smooth muscle cells, microglia, and neurons.[48,49] The

ability for cells to regenerate arginine from citrulline is necessary to ensure enough arginine for iNOS to maintain its role in homeostasis.

The availability of the essential cofactor $H_4B$ may also affect iNOS activity. $H_4B$ plays an essential role in the dimerization of iNOS to active enzyme.[30,50] Cell lines producing little $H_4B$ have low iNOS activity, but supplementation with $H_4B$ increases iNOS dimerization; the $H_4B$ precursor sepiapterin can also rescue iNOS activity.[51] Interestingly, during inflammation associated with wound healing, iNOS co-expresses and co-localizes with GTP-cyclohydrolase I, a key enzyme in the $H_4B$ biosynthetic pathway.[52]

### 3.2 Protein-protein interactions

Several proteins also interact with iNOS and regulate its activity. In the CNS, iNOS has been found in complex with a cytosolic protein called kalirin (mainly kalirin-7),[53] in brains of mice treated with LPS. Studies have found that kalirin interacts with iNOS monomers but not dimers. In kalirin-expressing cells, most iNOS exists as a heterodimer with kalirin. There is little monomeric iNOS, indicating that kalirin inhibits iNOS activity by preventing iNOS homodimerization.[54] Low expression of kalirin is correlated with increased iNOS activity in the hippocampus of Alzheimer's patients.[53] As excess NO produced during inflammation is neurotoxic, kalirin may be exerting neuroprotective effects by down-regulating iNOS.[53] In murine macrophages, iNOS interacts with a 110-kDa protein called NAP110 (NOS-associated protein 110). NAP110 and iNOS complexes are found in the spleens and peritoneal macrophages of IFN-$\gamma$ and LPS-treated mice. Co-expression of NAP110 and iNOS reduces the latter's activity by 90%, although the amount of iNOS protein does not change. Similar to kalirin, NAP110 inhibits iNOS activity by interacting with iNOS monomers and preventing the enzyme's dimerization. NAP110 may therefore be a regulatory switch to prevent NO levels from becoming toxic.[55,56]

iNOS has also been found to interact directly with Rho GTPase Rac 2, mainly through its oxygenase domain. Overexpression of Rac2 in RAW 264.7 cells potentiated LPS-induced iNOS activity.[56] Another study demonstrated that iNOS and Rac2 interact in human neutrophils. This interaction may play a key role in anti-microbial defense,[58] because cells treated with a Rac2 inhibitor generated less superoxide (which reacts with NO to produce toxic $ONOO^-$). These results suggest that Rac2 can enhance NO generation through iNOS.[57,58] Yoshida and Xia[59] found that the chaperone heat shock protein 90 (hsp90) can also increase iNOS activity in a dose-dependent fashion, and the two proteins co-immunoprecipitate from activated macrophages.

### 3.3 Auto-inactivation

NO itself may negatively regulate iNOS activity. In stimulated murine macrophages, iNOS activity is increased when hemoglobin, a NO scavenger, was added. Addition of the NO donors *S*-nitrosoacetylpenicillamine or *S*-nitrosoglutathione significantly inhibits iNOS activity, suggesting that NO may be involved in some negative feedback mechanism. Furthermore, iNOS activity cannot be restored in activated cells after NO donors are removed, an indication of the irreversible binding of NO to iNOS.[60] A kinetic study showed that the major mechanism for iNOS auto-inactivation is through *S*-nitrosation of the zinc-

tetrathiolate cluster (vide infra), which causes zinc loss, irreversible iNOS dimer dissociation, and subsequent loss of activity. Reducing agents can protect iNOS from this inactivation, suggesting *S*-nitrosation was indeed the major inactivation pathway.[61]

## 4. iNOS EXPRESSION AND SIGNALING PATHWAYS

iNOS expression is chiefly governed by transcriptional regulation,[62,63] which can differ depending on the cell type or species. In general, pro-inflammatory cytokines such as tumor necrosis factor alpha (TNF-α), interleukin-1 (IL-1), interferon-γ (IFN-γ), and LPS, first bind to receptors on the cell surface and activate kinases, leading to the phosphorylation of various intracellular proteins and subsequent activation of specific transcription factors, including nuclear factor κB (NF-κB) transcription factors such as nuclear factor 1 transducer and activator of transcription 1a (STAT-1a).[62] The active factors then translocate into the nucleus, where they bind to the promoter region of the iNOS gene and induce iNOS expression.

Different inducers trigger different signaling pathways. One of the major pathways involves activation of NF-κB, a major target of LPS, IL-1β, and TNF. In macrophages, LPS first activates a toll-like receptor (TLR4) on the cell membrane. The TLR4 complex contains several proteins including TNF-receptor associated factor 6 (TRAF6), myeloid differentiation primary response 88 (MyD88), and a kinase[64]. As NF-κB is a dimer in macrophages and is usually inactivated and bound by inhibitor κB (IκB), activation of this pathway induces a kinase cascade (one is via mitogen-activated protein kinase kinase, or MEKK), which leads to the phosphorylation of inhibitor of κB kinase (IKK) and subsequent phosphorylation of IκB. When phosphorylated, IκB is ubiquitinated and degraded in the proteasome, and NF-κB is released. NF-κB then translocates, interacts with the iNOS promoter, and triggers transcription of the iNOS genes[64,65] (Fig. 4, left).

By contrast, IFN-γ activates the JAK/STAT-1α pathway.[62,66,67] Briefly, inducing cells with IFN-γ causes the dimerization of IFN-γ receptor and activation of Janus kinases (JAK, such as JAK2), which then phosphorylates STAT-1α. STAT-1α itself then dimerizes and translocates to the nucleus, where it facilitates the synthesis of interferon regulatory factor 1 (IRF-1). IRF-1 then binds to the iNOS gene promoter and induces iNOS expression (Fig. 4, right).

## 5. DISTRIBUTION AND INDUCTION OF iNOS

iNOS was initially purified from murine macrophages.[14,68] Since then, different cell types, including hepatocytes,[69,70] smooth muscle cells,[71,72] chondrocytes,[73] glial cells,[74] astrocytes,[75] neurons,[76,77] and cardiac myocytes[78] from different species, have been found to induce iNOS when stimulated. Successful cloning of full length iNOS cDNA isolated from RAW 264.7 murine macrophages has provided the amino acid sequence of the protein and insight into its structure and regulation.[79,80] The murine macrophage iNOS amino acid sequence shares 51% homology with rat nNOS.[80]

Compared with other species, induction of iNOS in human cell types is limited. Human macrophages fail to produce sufficient amounts of NO under multiple different induction

conditions,[81] possibly the result of limited biosynthesis of $H_4B$, an essential iNOS cofactor, in human macrophages.[82] The first successful induction of human iNOS was in hepatocytes.[70,83] Human liver iNOS cDNA shares 80% sequence homology with murine macrophage iNOS. Successful induction of human iNOS has also been achieved in human chondrocytes using IL-1β.[73] cDNA cloned from this cell type shows 88% sequence similarity with murine macrophage iNOS, suggesting that iNOS structure and function is retained in different species and cell types.

In the CNS, iNOS is expressed in various glial cells, including astrocytes and microglia (the "macrophages of the brain"), in response to different stimuli.[74,75] Compared to glial cells, iNOS expression in neurons is very limited, but there is still evidence for its expression in these cells. For instance, treatment of different human neuroblastoma cell lines (widely used as models of human neuronal cells) with cytokines results in expression of iNOS genes.[84] Neuronal iNOS expression may also occur in Alzheimer's disease or following hypoxia or ischemia.[85,86]

TNF, IL-1β, IFN-γ, and LPS are the main inducers of iNOS expression. Combinations of these inducers can generate synergistic effects in certain cells[69,87] and are sometimes actually required to stimulate NO production, especially in nonimmune cells, where LPS only weakly induces iNOS.[88] For instance, in mouse skeletal myocytes, none of these inducers alone can induce iNOS expression. However, stimulation of cells with both IFN-γ and TNF or IL-1 (or with all three cytokines) results in significant NO production.[89] The stimuli and conditions that determine iNOS expression depend on the cell type and species. For example, LPS alone can stimulate iNOS expression in rat and mouse glial cells but not human ones.[74,90]

In addition to LPS and cytokines, invading viruses can also trigger iNOS induction in cells. Human immunodeficiency virus (HIV) induces iNOS expression in human glial cells,[90–92] and high iNOS expression is also detected in hepatocytes of patients infected with hepatitis B virus (HBV). Transfection of hepatocytes with HBV genes results in iNOS expression, which indicates the virus can upregulate iNOS expression in human liver cells.[93]

## 6. iNOS IN DISEASE STATES

Although iNOS is necessary for normal physiology, excessive amounts of NO as a result of overexpression or dysregulation of iNOS are implicated in human diseases. There are many studies showing that excessive NO plays a role in sepsis. In animal models, high NO production contributes to vasodilation, hypotension, and cardiovascular malfunction during endotoxemia and cytokine-induced shock.[65] High concentrations of nitrite and nitrate (the two stable products from NO production) are found in plasma of patients with septic shock,[94] and iNOS activity is also detected in gangrenous tissues from sepsis patients, including fat, muscle, and arteries, but not skin.[95] Additionally, increased iNOS activity is found in lung macrophages from patients with acute respiratory distress syndrome following sepsis.[96] Because of the lethality of septic shock, this indication has spurred the development of numerous iNOS inhibitors.

High iNOS expression has also been found in human patients infected with different pathogens, particularly HIV,[97] *Mycobacterium tuberculosis*,[98] and *Plasmodium falciparum*.[99] Indeed, both HIV and tuberculosis infection induce iNOS expression in brain-derived cells, and in the former case, this NO may contribute to the neuronal damage of AIDS-related dementia.[92,97] iNOS induction may also be linked to diabetes and obesity-associated insulin resistance, as it is known that iNOS is expressed throughout insulin-sensitive tissues, and obesity increases iNOS levels in muscle, adipose tissue, and vasculature.[100,101] Studies in rat models show that rat pancreatic islet cells are very susceptible to NO toxicity[102] and iNOS overexpression is found in pancreatic macrophages of prediabetic rats.[103] iNOS' role in insulin resistance may in part be through *S*-nitrosylation or tyrosine nitration of insulin signaling protein.[104,105]

High levels of iNOS expression are also found in many cancerous tumors, including, colon, bladder, breast, and lung cancer.[41] The role of iNOS in cancer is somewhat complex, as it has both tumor promoter and suppressor effects depending on the tumor and local environment. Nitrosation or nitrosylation (by NO and its products) in cancer cells can contribute to increased proliferation, metastasis, and even drug resistance.[41] Most recently, high iNOS expression was correlated with poor survival in triple-negative breast cancer patients.[106] In addition, NO produced by iNOS within the tumor itself can inhibit proliferation of T-lymphocytes (as seen in rat colon cancer models), which could explain how tumors can suppress host immune functions.[107]

Various studies have also indicated that iNOS (both centrally and peripherally expressed) may play a role in the development and sensation of inflammatory and neuropathic pain. Meller *et al.* found that LPS and cytokines induce thermal hyperalgesia (hypersensitivity to pain), possibly mediated via induction of iNOS in spinal cord glial cells.[108] Similarly, in animal models of neuropathic pain, iNOS upregulation contributes to local inflammatory reactions.[109] Recently, even cancer-associated pain was found to be associated with upregulated iNOS (and nNOS) in mice with bone cancer.[110] As such, treatment of pain has also been a major driving force for development of iNOS inhibitory therapeutics.

The normal brain does not appear to express much iNOS, but following trauma, inflammatory damage or stimuli, or hypoxia (such as a from a stroke or ischemic event), iNOS expression in activated glial cells and neurons can occur.[74,86,111] Some of these iNOS-producing cells build up around damaged sites in the brain, which could indicate some inflammatory link between NO originating from glial cells and neuronal injury.[74] iNOS has been found in the brains of patients with Alzheimer's disease,[85,112] and high levels of iNOS have also been observed in multiple animal models of Parkinson's disease.[113] However, iNOS knock-out mice genetically cross-bred with transgenic mice bred to produce beta-amyloid plaques showed a much higher incidence of Alzheimer's phenotypes than the transgenic mice bred to produce beta-amyloid plaques alone, suggesting iNOS could be protective and its inhibition might be detrimental.[114] There is still some uncertainty, however, as to whether iNOS expression and activity are a direct cause of neuronal damage or are merely associated with certain stages of neurodegenerative disorders (i.e., correlation vs. causation), or might even be protective in some cases.

# 7. DEVELOPMENT OF iNOS INHIBITORS

## 7.1 Assaying for iNOS activity and inhibition

Several in vitro assays are commonly used to measure iNOS activity during the development of iNOS inhibitors. Isolated enzyme-based systems are often initially used to determine the extent of iNOS inhibition. One common method is to measure the conversion of radiolabeled ($^{3}$H or $^{14}$C)-L-Arg to L-citrulline[115] using human or murine iNOS, although non-radioactive enzyme-based methods, such as the hemoglobin capture assay (where the oxidation of hemoglobin to methemoglobin by NO is measured spectrophotometrically) or are also employed.[115] Cell-based systems (especially for dimerization inhibitors), where a macrophage cell line (such as RAW 264.7)[116] is stimulated to express iNOS by action of added cytokines, have also been used. In these systems, NO production is monitored, for example, via the addition of Griess reagent to cell lysates, which detects nitrite production.

The most common in vivo assay for iNOS inhibition is the rodent endotoxemia model, which is roughly analogous to the systemic inflammatory phenotype observed in sepsis[117]. In this assay, an endotoxin (usually LPS) is administered to a rodent, which causes systemic inflammation with concomitant increases in cytokines, iNOS, and NO production, which can be monitored via plasma nitrate/nitrite levels.

Formalin or carrageenan models are often used to model inflammatory pain. A small amount of formalin solution is injected into the paw of a rodent to cause local inflammation[118], and the frequency or duration of pain behaviors (flinching or biting and licking the paw) are measured in the presence or absence of the inhibitor of interest. Likewise, carrageenan injection[119] causes localized edema, pain, and hyperalgesia. To assess iNOS' possible role in neuropathic pain, surgical models such as the Chung model (pain is induced through surgical ligation of the spinal nerve)[120] or the chronic constriction injury model (ligation of the sciatic nerve)[121] are used. As iNOS inhibitors have also been investigated for arthritis, the adjuvant-induced arthritis model[122] is employed to assess their efficacy. An adjuvant (such as Freund's Complete Adjuvant) is administered to an animal to induce an arthritic phenotype. In the presence or absence of a drug, the animal is observed for arthritic symptoms and clinically scored; postmortem dissection and examination of the affected joint components are also sometimes employed.

## 7.2 Competitive iNOS inhibitors

There are two main modes of iNOS inhibition that act directly on the enzyme. First, the majority of iNOS inhibitors discovered are directly competitive with L-Arg (often acting by coordinating with the active-site L-Glu), and they either reversibly inhibit L-Arg binding or compete with L-Arg and then irreversibly inactivate the enzyme in a time-, concentration-, or NADPH-dependent manner. The activity and selectivity of iNOS inhibitors discussed in this section is summarized in Table 1.

Some of the earliest competitive iNOS inhibitors reported were arginine derivatives: L-nitroarginine, its more bioavailable methyl ester (L-NAME, **1**, Fig. 5), and L-$N^{\omega}$-monomethylarginine (L-NMMA, **2**).[123] Although **2** is both a competitive inhibitor and slow, inactivator of iNOS (possibly through a hydroxylated intermediate and heme loss),[124] simple

arginine derivatives have little selectivity for iNOS over both of the other isoforms: **2** inactivates both iNOS and nNOS, and both **1** (in its hydrolyzed acid form, as L-NAME is only a weak inhibitor).[123] and **2** are actually selective for eNOS. As such, both nitroarginines and **2** cause hypertension when administered to rats or humans,[96,125] likely the result of inhibition of eNOS-mediated vasodilation.

The amidine $N^5$-(1-iminoethyl)-L-ornithine (L-NIO, **3**), is a competitive, mechanism-based inactivator[126,127] with ~1.8-fold selectivity for murine iNOS over rat nNOS,[128] but little selectivity over bovine eNOS.[123] In the rat endotoxemia model, László *et al.*[129] examined the ability of iNOS inhibitors to prevent endotoxin-induced colonic microvessel damage and albumin leakage caused by excess NO. When administered during the period where iNOS was induced (~4 h past LPS insult), **3** significantly attenuated colonic leakage, but when administered earlier with LPS, **3** (along with **2**) actually exacerbated vessel damage, presumably via inhibition of eNOS. Interestingly, bulky analogues of **3**[130] inactivate iNOS via a different mechanism than **3** (which induces heme loss) although they are less potent and their selectivity was not reported.

Aminoguanidine (**4**) is a time-dependent, competitive, mechanism-based inactivator of all NOS isoforms that may bind preferentially to iNOS.[126] Corbett *et al.* reported that **4** was around 40 times less effective at raising mean arterial blood pressure in rats than **2**, suggesting some selectivity for iNOS over eNOS in vivo.[131] Misko *et al.*[132] confirmed selectivity for iNOS over nNOS in both cell-based and cell-free extracts, but AstraZeneca later reported little selectivity.[133] In vivo, **4** shows a variety of iNOS-mediated effects, including the ability to ameliorate negative cardiovascular changes in endotoxin-challenged rats, improve survival in the mouse endotoxemia model,[134] and delay disease onset and severity in a murine autoimmune encephalomyelitis model (of multiple sclerosis.[135]

$N^6$-(1-Iminoethyl)-L-lysine (L-NIL, **5**), a competitive inactivator like **3**,[136] was reported to be more potent in vitro ($IC_{50}$ = 3.3 μM for murine iNOS) than **2**, **3**, and nitroarginine, with 28-fold selectivity for iNOS over nNOS.[128] Promisingly, **5** reduces early osteoarthritis progression in dogs without toxicity, possibly through reduction of apoptosis in cartilage.[137] Nonetheless, **5** has undesirable physical properties, which prompted Hallinan and colleagues[138] to devise a prodrug form of **5**, the tetrazoleamide L-NIL-TA (or SC-51, **6**). While inactive in vitro against human iNOS, the drug is orally bioavailable and hydrolyzes to **5** in vivo, and its activity is comparable to **5** in the LPS endotoxin model, the carrageenan inflammation model, and the adjuvant-induced arthritis model in rats without raising blood pressure. Interestingly, **6** also reduces exhaled NO in both asthmatic and healthy human patients,[139] but it does not appear to have been further investigated clinically for this indication despite iNOS' implication in asthma pathology (see asthma discussion in section on New Directions for iNOS Therapeutics).

Two other amino acid amidines of note are **7** and **8** (Fig. 6, developed by Glaxo-Wellcome; known as GW274150 and GW273629, respectively). These two compounds are potent ($K_d$ <90 nM, $IC_{50}$ <10 μM), selective (>80-fold i/n, >100-fold i/e), and time- and NADPH-dependent inhibitors (or inactivators) of both human and rodent iNOS. Effective at inhibiting plasma nitrate/nitrite increase in the mouse LPS model, these compounds also do not inhibit

nNOS and eNOS in tissue slices or in vivo.[7] Compound **7** showed analgesic activity in several rat pain models (adjuvant-induced paw inflammation and chronic constriction injury[140] and reduced carrageenan-induced lung inflammation in rats in a dose-dependent fashion (with effects beginning at 2.5 mg/kg).[141] Compound **7** progressed into Phase II clinical trials for rheumatoid arthritis and migraine. Although it showed some ability to reduce synovial thickness and vascularity in Phase IIa patients,[142] it did not progress further. Additionally, **7** showed no ability to prevent migraines when dosed prophylactically;[143] clinical development of **7** appears to have ceased ca. 2012.

A related analogue, the selective, irreversible inhibitor cindunistat (SD6010, **9**)[144] was investigated by Pfizer for the treatment of osteoarthritis. Although it showed good efficacy in rodent models of inflammatory, neuropathic, and osteoarthritis-related pain,[145] **9** failed to reduce pain and joint-space narrowing in human patients, although other joint changes were not measured, and the drug was well-tolerated.[146]

Various ureas, thioureas, and isothioureas have also been investigated as iNOS inhibitors. Southan and colleagues published the first report of simple isothioureas inhibiting iNOS in 1995,[147] but these molecules were still under investigation as of 2016. Recently, kynurenamine urea (such as **10**)[148,149] and thiourea (**11**) were shown to be both iNOS or dual iNOS and nNOS inhibitors with low eNOS inhibition and cytotoxicity.[149] Although their potency is modest, molecular modeling supports the hypothesis that they are competitive with L-Arg. Bisisothiourea **12** (known as PBITU)[150] is a very potent human iNOS inhibitor ($K_i$ = 47 nM) with 190-fold selectivity for iNOS over eNOS, but it showed high acute toxicity in rodents.

Optimization of **12** led to **13** (1400W) a very potent and selective (~5000-fold i/e and >250-fold i/n) iNOS inhibitor.[151] Mechanistically, **13** displays only weak competitive inhibition of eNOS and nNOS, but iNOS inhibition was found to be time- and NADPH-dependent, suggesting possible inactivation (with a measured $K_d$ of 7 nM). Later studies showed that **13** was not modified by the enzyme or utilized as a substrate, but inactivated iNOS via a mechanism consistent with loss of heme as biliverdin (as **3** does).[127,152] Administration of **13** reversed LPS-induced vascular injury in rats,[151] and **13** also showed efficacy in animal pain models. Recent studies suggest that 1400W's anti-inflammatory effects may be more complex than just iNOS inhibition, because in rat neuropathic pain models treated animals actually had increased levels of anti-pain cytokines.[153] SAR studies of similar benzamidines performed by Amoroso and co-workers[154] revealed that truncated compound **14** was more potent than **13**, but less selective, suggesting that the aminomethyl group of **13** disfavors binding to eNOS and is necessary for full selectivity.

Although Merck, AstraZeneca, Searle/Pharmacia, and various academic groups have investigated both amino acid and aromatic amidines as competitive iNOS inhibitors, cyclic amidines (Fig. 7) are the most studied.[133] Among other 2-iminoazaheterocycles investigated by Moore and co-workers, 2-iminopiperidine (**15**) was the most potent against human iNOS ($IC_{50}$ = 1 μM). It showed ability to inhibit plasma NO increases in LPS-challenged rats when dosed orally, but did not have much selectivity over nNOS or eNOS.[155] Ono Pharmaceutical Company discovered another iminopiperidine (ONO-1714, **16**) with very

potent iNOS inhibition ($K_i$ = 1.88 nM) but weak selectivity (10-fold) for iNOS over eNOS.[156] This compound also was later revealed as a very potent nNOS inhibitor.[157] Compound **16** reduced lung injury in endotoxin-challenged rabbits[158] and decreased lactic acidosis and hypotension in a canine endotoxic shock (LPS) model, but caused increased vascular resistance at higher doses in the latter animal model, possibly because of eNOS inhibition.[159] 2-Iminopyrrolidines (such as **17**) also showed some promise as iNOS inhibitors (human iNOS $IC_{50}$ = 0.25 μM) and activity in rodent endotoxemia models[160] and had much higher i/e selectivity than **15** and **16**, but low i/n selectivity (13-fold). Hydroxylation (as in **18**) afforded a 3-fold increase in i/e selectivity and a 2.5-fold increase in i/n selectivity.[161]

In 2001, AstraZeneca reported that 3,4-dihydro-1-isoquinolinamines (especially 3-phenyl-3,4-dihydro-1-isoquinolinamines) inhibited iNOS.[162] Compound **19** had a human iNOS $IC_{50}$ of 160 nM, with i/n selectivity of 100-fold and i/e selectivity of approximately 1,000-fold. A derivative of **19**, spirocyclic amide **20** (AR-C102222)[163] maintains high i/e selectivity but has decreased i/n selectivity. Nonetheless, this compound displays high in vivo potency without adverse cardiovascular issues and had protective effects in rat models of adjuvant-induced arthritis. Compound **20** also had beneficial effects in a variety of mouse pain and inflammation models (ear inflammation and spinal nerve and hindpaw injury).[164]

Although X-ray crystal structures of iNOS have been available for over 20 years,[28,165] most iNOS inhibitor design is not explicitly structure-based. A notable exception is the anchored plasticity method from AstraZeneca.[166] In this method, a combination of X-ray crystallography and mutagenesis studies was used to elucidate the presence of an iNOS-specific pocket that opened upon inhibitor binding as a result of conformational changes in the enzyme. This area was then used to design new compounds, using a strategy where a known functional group capable of iNOS inhibition (such as an amidine) was “anchored” in the binding site, and then other portions were elaborated outwards to reach this induced-fit specificity pocket. Through this method, compound **21**, with an $IC_{50}$ of 400 nM (human iNOS) and n/e selectivity of 200, was designed. In the X-ray crystal structure of **21** bound to murine iNOS (Fig. 8), the thiophenecarboxamidine of **21** mimics L-Arg and forms a salt-bridge with the catalytic glutamate residue.

2-Aminopyridine is a more recently investigated L-Arg mimic for both iNOS and nNOS (Fig. 9). AstraZeneca reported that simple aminopyridines can antagonize L-Arg in iNOS,[167] although most of these compounds are nonselective, weakly selective, or show lower activity in vitro and in vivo than the corresponding saturated cyclic amidines (such as **15**). 4-Methylaminopyridine (**22**, 4-MAP)[167,168] is a nonselective iNOS inhibitor that shows activity in rodent endotoxemia and pain models, although the dose required for analgesic effects is lower than those needed for efficacy in the endotoxic shock model, suggesting some of its analgesic properties are likely the result of nNOS inhibition.[168]

The disubstituted aminopyridine (**23**) is about six times more potent than **22** and has improved selectivity,[167] but more complex aminopyridines are generally more effective. Through optimization, AR-C133057XX (**24**) was discovered.[169] This piperidine amide derivative is potent in vivo and in vitro ($IC_{50}$ = 71 nM against human iNOS) and extremely selective for iNOS over eNOS (<20% eNOS inhibition at 100 μM). X-ray crystallography

revealed that the aminopyridine of **24** flips over 180° relative to that of **22**, which the authors propose allows binding of the piperidine and benzonitrile to additional iNOS-selective regions.

An unconventional pyridine derivative is imidazopyridine **25** (BYK191023), which was discovered in 2005 in a high-throughput screen.[170] Two years later it was determined to be a competitive, time-dependent, irreversible inhibitor in the presence of NADPH, likely causing the loss of heme.[171] Highly selective for iNOS (in enzyme assays, cells, and isolated organ systems), **25** was also tested in several animal models. In addition to reduction of plasma nitrite/nitrate and maintenance of blood pressure in LPS-challenged rats,[172] **25** improved organ blood flow and reduced pulmonary hypertension and acidosis in septic sheep (relative to sheep treated with norepinephrine), but the drug did not increase overall survival.[173]

### 7.3 Dimerization inhibitors

The second mechanism upon which inhibitor development has focused is inhibiting formation of an active iNOS dimer. As the dimer interface is located at the far end of the active site, an inhibitor that anchors in the active site and blocks dimerization is desirable. It has long been known that imidazoles (Fig. 10) can coordinate the heme iron of NOS isoforms. While smaller imidazoles stabilize iNOS dimer formation, bulky imidazoles (such as the antifungal clotrimazole (**26**)) prevent dimer assembly.[174]

Following this discovery, pyrimidinylimidazoles (such as **27**) were discovered by combinatorial chemistry at Berlex Bioscience.[175] These compounds bind with high affinity to human iNOS monomers ($K_i$ of 2.2 nM for **27**) and show activity in rodent LPS models. X-ray crystallography of **27** bound to a murine iNOS monomer missing the N-terminal 114 residues (Fig. 11) suggested that these compounds act by coordinating iron, adopting a U-shaped conformation, and displacing the Glu371 residue from the active site. Disruption or disordering of this residue's helix (helix 7a) then subsequently perturbs the dimerization interface. Mechanistic studies (spectroscopy and competition binding) performed by Blasko *et al*[176] later confirmed that pyrimidinylimidazoles do act by coordinating the heme center of iNOS monomers and allosterically blocking dimerization, and it was proposed that the observed selectivity reflects different dimer assembly kinetics in eNOS and nNOS (vs. iNOS). It was shown more recently[177] that pyrimidinylimidazoles can also break up active dimers into monomers in cells and that the monomer-inhibitor complex is irreversible and cannot be converted back into active dimers by added L-Arg or $H_4B$.

By X-ray crystallography, homoproline compound **28** also prevents dimerization by displacing iNOS' helix 7a[178]. This compound is orally bioavailable and is an excellent inhibitor in cells ($IC_{50}$ = 290 pM), presumably acting by binding to nascent iNOS monomers as, or shortly after, they are formed. Compound **28** also reduced clinical arthritis scores in adjuvant-induced arthritic rats.

In addition to Berlex Biosciences' work, Kalypsys Inc. was also active in the area of iNOS dimerization inhibitor development. In 2009, an ultra high-throughput cell-based screen was performed on non-L-Arg and non-imidazole compounds (the latter out of concern over cytochrome inhibition), and quinolinone hit **29** was identified.[179] Optimization was

performed to improve murine iNOS activity (for animal use), and new leads **30** (KLYP596) and **31** were found to have improved potency and selectivity, with **30** having an $EC_{50}$ of 11 nM and the higher selectivity of the two (i/e of 2300 and i/n of 210) in cell-based assays. Gel electrophoresis and immunoblotting of enzyme isolated from treated cells revealed dose-dependent loss of dimeric iNOS.[180] Biochemical characterization revealed that inhibition was insensitive to addition of L-Arg and $H_4B$, but binding of **30** did not result in spectral shifts consistent with imidazole-like heme coordination. Although **30** and **31** have good oral bioavailability, they have high clearance and a short half-life in vivo. Nonetheless, both compounds reduce plasma nitrate levels in LPS-challenged mice and reduce pain behaviors in mouse formalin and chronic nerve constriction injury models.[179]

To improve pharmacokinetics, researchers at Kalypsys also investigated quinolinone-benzimidazoles and imidazopyrazines as conformationally restricted analogues of **29**, and after SAR analysis, cyclobutyl analogue **32** (KD7332) was discovered.[181] A potent dual iNOS/nNOS inhibitor, **32** displayed reduced clearance in vivo, and showed anti-pain activity in the mouse formalin model and the Chung model of neuropathic pain, with a large therapeutic index. Another potent ($IC_{50}$ = 3 nM) and selective (nNOS $IC_{50}$ = 2.4 μM, eNOS = >30 μM) Kalypsys molecule (**33**, KD7040)[182,183] was described as being a skin-permeable selective iNOS dimerization inhibitor. It was investigated (Phase I) for topical use in the treatment of post-herpetic neuralgia, but its development appears to have ceased ca. 2008.

## 8. CLINICAL FAILURES

The classical indications for iNOS inhibition are sepsis (systemic inflammation) and pain. However, attempts to treat sepsis and septic shock with iNOS inhibitors in a clinical setting have been met with limited success. Although some beneficial effects have been shown, nonselective iNOS inhibition (such as with **2**) can actually increase cardiac dysfunction and mortality in septic shock patients.[184] It is proposed that eNOS could have some protective role in sepsis (as has been demonstrated in murine eNOS overexpression studies)[185] and thus nonselective inhibition could be harmful. Selective iNOS inhibition results in fewer side effects, but is still controversial for this indication.[173,186] Additionally, iNOS knockout mice (or mice treated with **1**, **2**, or **3**) are not protected from developing endotoxemic phenotype;[187] this effect has also been reported to occur in liver cells).[40] Bredan and Cauwels also suggest that agents that scavenge NO or mediate the downstream effects of NO could be more effective than iNOS inhibition itself .[188]

Despite efficacy in animal models, no iNOS inhibitors have been approved for pain treatment; arthritis trials have repeatedly failed, and anti-nociceptive activity in animal models does not translate to humans in clinical trials. First, it is possible that the role of NO in pain is more complicated than simply “too much NO is bad” (as is proposed to be the case for sepsis). Second, it is possible that iNOS inhibition could have real benefits, but the development of pain therapeutics (*not* exclusive to iNOS) is fraught with failure, in part because of possible assay limitations, and in part because pain is subjective and both affects - and is affected by – other complex behaviors in both animals and humans.[189]

Another area where iNOS inhibition seems to have failed is treatment of cardiogenic shock related to myocardial infarction. Early studies suggested that iNOS plays a major role in heart failure, as upregulation of iNOS in mouse heart cells results in cardiac death.[190] However, in the best-known clinical trial for cardiogenic shock in infarction patients (the TRIUMPH trial),[191] compound **2** failed to reduce severity of heart failure or mortality and also resulted in hypertension. It was hypothesized that lack of selectivity[192] or too low a dose[193] could have been responsible for these failures. A recent study (2015) using resuscitated swine found that neither global NOS inhibition (using **1**) nor more selective iNOS inhibition (using **4**) improves survival or myocardial dysfunction following ventricular fibrillation in cardiac arrest (when iNOS levels increase).[194] Additionally, the observation that nonselective inhibition adversely affects blood pressure and cardiac function in this study also suggests that NO may be actually important for proper recovery from cardiac events.

## 9. NEW DIRECTIONS FOR iNOS THERAPEUTICS

Despite the focus on septic shock and various types of pain, it is conceivable that other diseases with a sizeable inflammatory component could benefit from iNOS inhibitors. Indeed, many studies indicate that iNOS inhibition could have broader therapeutic use in other diseases, including neurologic disease, diabetes, cancer, and respiratory disease; these uses are summarized briefly below.

Although the NOS isoform most investigated as a target for anti-neurodegenerative agents is nNOS,[195] evidence suggests that iNOS inhibition also might be beneficial for treating or preventing neurodegenerative disorders, such as Parkinson’s disease or the neuronal damage associated with stroke. For example, iNOS silencing (with siRNA) reduces the Parkinsonism symptoms in animals treated with the neurotoxin 6-OHDA.[196] Compound **4** shows beneficial effects in animal models of stroke, such as ability to reduce infarct volume in hypoxic-ischemic rat pups[197] and in adult rats,[198] although iNOS inhibition is believed to be responsible for only part of the observed effects in the latter study. Another mechanism, potentially polyamine oxidase inhibition by **4**, also may be responsible. Selective iNOS inhibition (such as with **7**) has also been found to be neuroprotective, preventing neuroinflammation in the 6-OHDA model of Parkinsonism in rodents at 10 mg/kg, although higher doses seem to have little effect.[199] The naturally occurring flavonol quercetin (**34**, Figure 12) prevents both 6-OHDA-induced apoptosis in neuron-like PC12 cell cultures and neuronal death in zebrafish. This phenomenon was only observed in early zebrafish larvae, suggesting that this compound may not cross the blood-brain barrier effectively. Quercetin also lowers iNOS expression in PC12 cultures (and it is proposed that some of its neuroprotective effect may come from reduction of iNOS expression), but **34** also can lower the expression of other pro-inflammatory cytokines; therefore, its effects may be multifaceted.[55]

A 2014 report[200] discusses a series of natural product-like compounds discovered via high-throughput virtual screening. One compound (bis-indole **35**) is proposed to be a competitive inhibitor of NO production in stimulated macrophages. Compound **35** also displayed partial

neuroprotective effects in zebrafish larvae treated with the neurotoxin MPTP, although these effects were not correlated specifically with iNOS inhibition.

iNOS inhibition has also been investigated more recently as a target of interest in epilepsy. Uzüm *et al.* demonstrated in rats that epilepsy (represented by long-term clonic-tonic seizures), like sepsis, is associated with systemic inflammation (including increased iNOS levels in the liver and kidney).[201] Other studies have shown iNOS inhibition could be beneficial in epilepsy. For example, aminoguanidine (**4**) suppresses the development of chemically induced kindled and spontaneous recurrent seizures in mice in a dose-dependent fashion.[202] Furthermore, a selective iNOS inhibitor, **13**, reduces the development of temporal lobe epilepsy in rats treated with the neurotoxin kainate.[203] In this study, treated animals had reduced neuronal hyperexcitability (epileptiform spiking) and displayed a 90% reduction in spontaneous recurrent seizures (relative to control) in the six-month period following the kainate insult.

Overall, iNOS' role in the brain may be more complicated than originally believed. Recent studies suggest iNOS may also play some role in depression. Montezuma *et al.* report that both iNOS deletion and selective iNOS inhibition (using either **4** or **13**) reduce immobility time in mice in the forced swimming test in the same way that antidepressant drugs do, but nNOS-specific inhibitors (at the same doses relative to their $K_i$ values) do not.[204] Compound **13** also exhibits an ability to reverse stress-induced depressive behaviors in mice consistent with prior results that indicate chronic stress may lead to an increase in general inflammation.[205]

A full summary of the role of iNOS in cancer is beyond the scope of this review,[41,206] although iNOS inhibitors have demonstrated a variety of chemopreventive and anticancer effects in animal models. Both **4** and **6**, for example, reduce the incidence and number of preneoplastic lesions in rats fed a selective colon carcinogen; administration of the iNOS inhibitor with the COX-2 inhibitor celecoxib further enhanced chemopreventive potential, suggesting that overexpressed iNOS could be regulating inflammation through COX-2.[207] Positive effects from iNOS inhibition were also observed in mice implanted with human melanoma xenografts; compound **5** slowed tumor growth and showed synergistic activity when administered with cisplatin.[208] Similarly, **5**, **22**, and a variety of isothioureas prevented transformation of rat tracheal cells into cancer cells.[209] Both iNOS knockdown and the use of compounds, **1**, **2**, and **13** inhibit breast-derived cancer cell proliferation, cancer stem-cell self-renewal, and migration in vitro. Compounds **1** and **2** also reduced tumor volume and lung metastases in triple-negative breast cancer mouse xenograft models, where their activity also synergized with docetaxel.[106]

iNOS inhibition has also been investigated for the treatment of the chronic, low-grade inflammation associated with type II diabetes, obesity-associated insulin resistance, and related conditions. Perrault and Marette reported that iNOS-knockout mice, while not protected from diet-induced obesity, are protected from associated insulin resistance and show improved glucose tolerance and normal glucose uptake in muscle.[210] Noronha and colleagues observed similar results on insulin tolerance, but obese iNOS knockout mice still were hypertensive with increased vascular reactive oxygen species.[100] Similarly, **5** improved

insulin sensitivity and fasting hyperglycemia in obese and diabetic mice (which have iNOS overexpressed in the liver, muscle, and adipose tissue.[211] Obesity can also impair lymphatic function (possibly via the accumulation of perilymphatic inflammatory cells that produce iNOS), and treatment of obese mice with **13** resulted in decreased perilymphatic inflammatory cell localization and modest improvements in lymphatic function.[212]

Finally, iNOS inhibition has potential therapeutic indications in the treatment of certain respiratory conditions. Mice lacking iNOS (but not eNOS) are protected from tobacco smoke-induced chronic obstructive pulmonary disorde.[213] Treatment with **5** reversed eight months of tobacco-induced lung injury after three months of administration. According to Nathan,[214] for this and other conditions, iNOS may be “beginning to smoke.”

## 10. CONCLUDING REMARKS

iNOS has been shown to have both beneficial and detrimental consequences. NO produced by iNOS is essential for the normal inflammatory response, while dysregulation of iNOS is implicated in a variety of chronic and acute diseases. Recent advances in structural characterization and new insights into regulation of iNOS expression have allowed the design and development of highly selective and potent iNOS inhibitors. Although iNOS inhibitors show significant promise in animal models for septic shock, pain, and other conditions, to date they have failed in clinical trials. Further efforts in the field should focus on understanding the complex role of iNOS in biological systems (both its harmful and protective roles) and refinement of both animal models and clinical trials for inflammatory diseases. New indications that iNOS inhibition could be useful for include treating cancer, neurodegenerative diseases, and depression (among others); these indications have revitalized the field and may lead to novel therapeutic avenues.

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## Biography

## Abbreviations Used

| | |
|---|---|
| **6-OHDA** | 6-hydroxydopamine |
| **CaM** | calmodulin |
| **CAT** | cationic amino acid transporter |
| **cDNA** | complementary DNA |
| **cGMP** | cyclic guanosine monophosphate |
| **CNS** | central nervous system |
| **COX-2** | cyclooxygenase 2 |

| | |
|---|---|
| **$EC_{50}$** | concentration of drug that gives half maximal response |
| **eNOS** | endothelial nitric oxide synthase |
| **EPR** | electron paramagnetic resonance |
| **FAD** | flavin adenine dinucleotide |
| **FMN** | flavin mononucleotide |
| **GTP** | guanosine triphosphate |
| **$H_4B$** | (6*R*)-5,6,7,8-tetrahydrobiopterin |
| **HBV** | hepatitis B virus |
| **HIV** | human immunodeficiency virus |
| **HSP** | heat shock protein |
| **$IC_{50}$** | concentration to achieve 50% inhibition |
| **i/e** | selectivity of a compound for iNOS versus eNOS |
| **i/n** | selectivity of a compound for iNOS versus nNOS |
| **IFN-γ** | interferon γ |
| **IRF-1** | interferon regulatory factor 1 |
| **IκB** | inhibitory κB |
| **IKK** | inhibitor of IκB kinase |
| **iNOS** | inducible nitric oxide synthase |
| **IL-1** | interleukin 1 |
| **JAK** | Janus kinase |
| **$K_d$** | dissociation constant |
| **$K_i$** | equilibrium constant for dissociation of an enzyme–inhibitor complex (used as a measure of potency where lower numbers indicate higher potency) |
| **L-Arg** | L-arginine |
| **L-NOHA** | Nω-hydroxy-L-arginine |
| **LPS** | lipopolysaccharide |
| **NADPH** | reduced nicotinamide adenine dinucleotide phosphate |
| **NAP110** | NOS associated protein 110 |
| **NF-κB** | nuclear factor κB |

| | |
|---|---|
| **nNOS** | neuronal nitric oxide synthase |
| **NO** | nitric oxide |
| **NOS** | nitric oxide synthase |
| **ONOO** | peroxynitrite anion |
| **SAR** | structure-activity relationship |
| **siRNA** | small interfering RNA |
| **STAT-1α** | signal transducer and activator of transcription 1α |
| **TNF** | tumor necrosis factor alpha |

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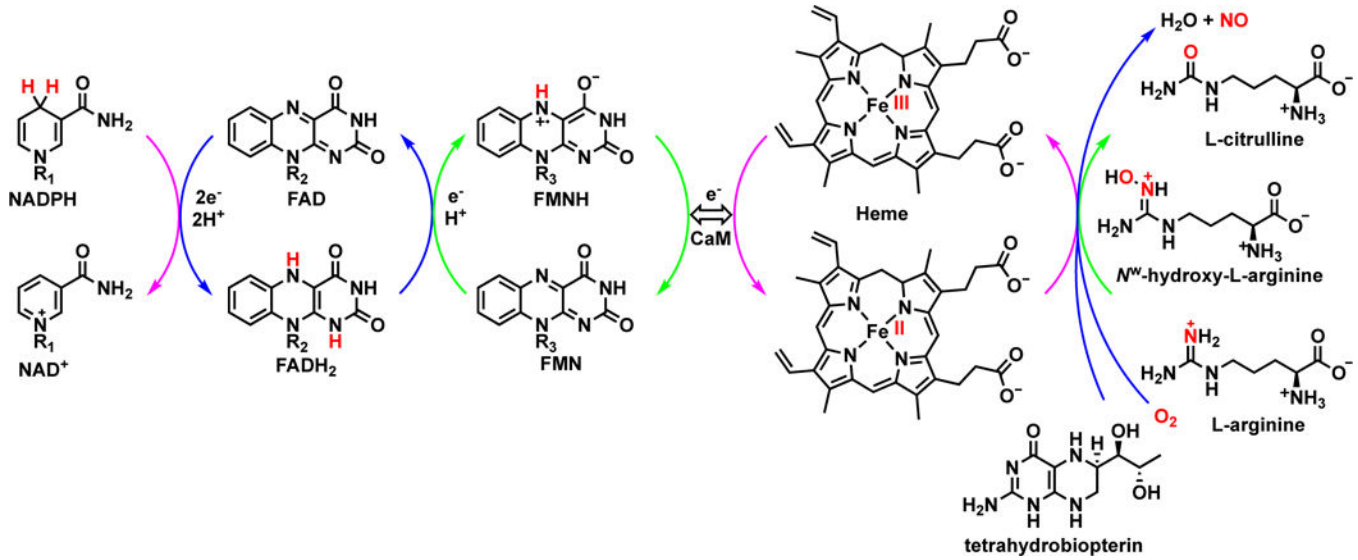


**FIGURE 1.**

Schematic representation of electron transfers from NADPH to heme through FAD and FMN (via tetrahydrobiopterin). The double-headed arrow indicates a conformational change to bring FMN into proximity with heme. The exact catalytic mechanism is not shown. Figure adapted with permission[195]

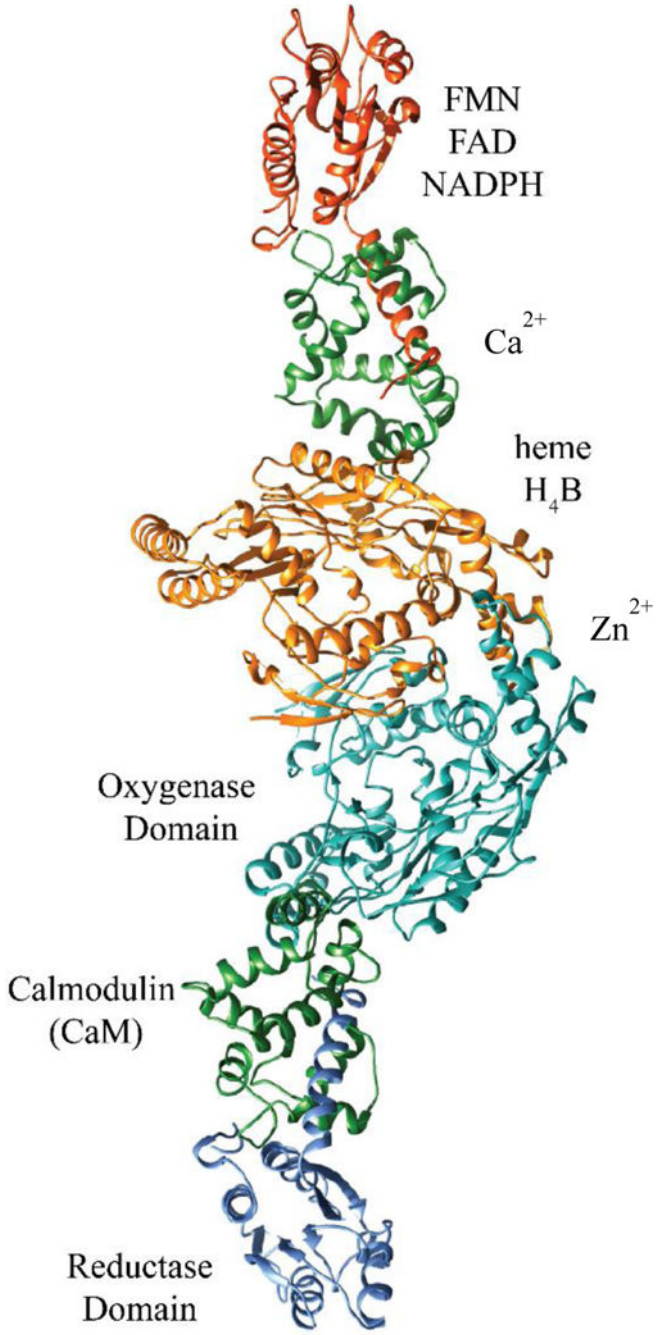


**FIGURE 2.**

Assembled dimeric iNOS X-ray crystal structures organized based on cryo-EM data, from PDB IDs 3HR4 and 4CX7.[29,38] The reductase domain shown is a 212-residue fragment of the full-length domain. Domains are annotated on the right side, cofactors on the left. The dimer interface is located between the orange- and cyan-colored domains.

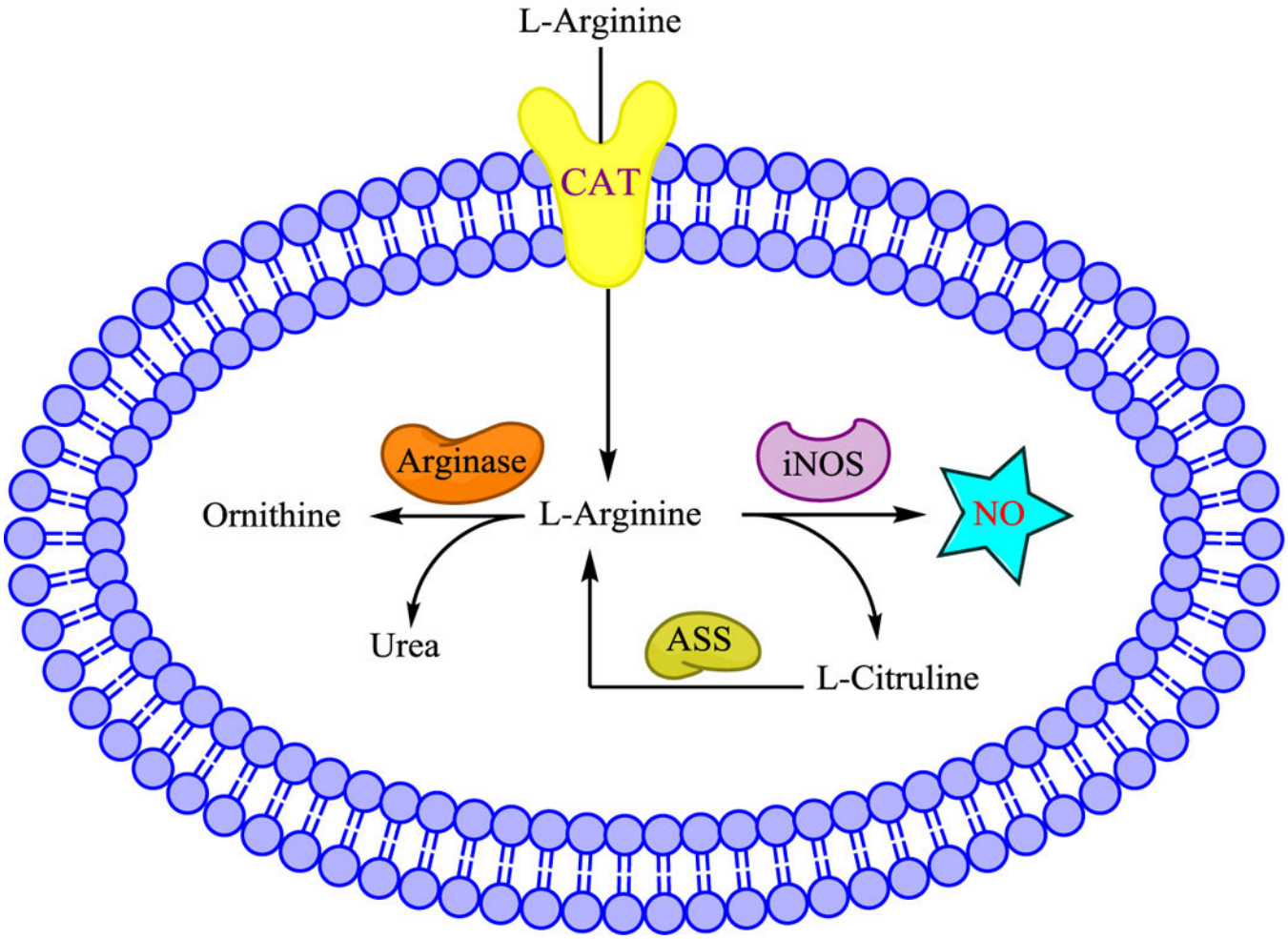


**FIGURE 3.**

Regulation of iNOS activity by the availability of its substrate L-Arginine. CAT: cation amino acid transporters. ASS: Arginosuccinate synthase.

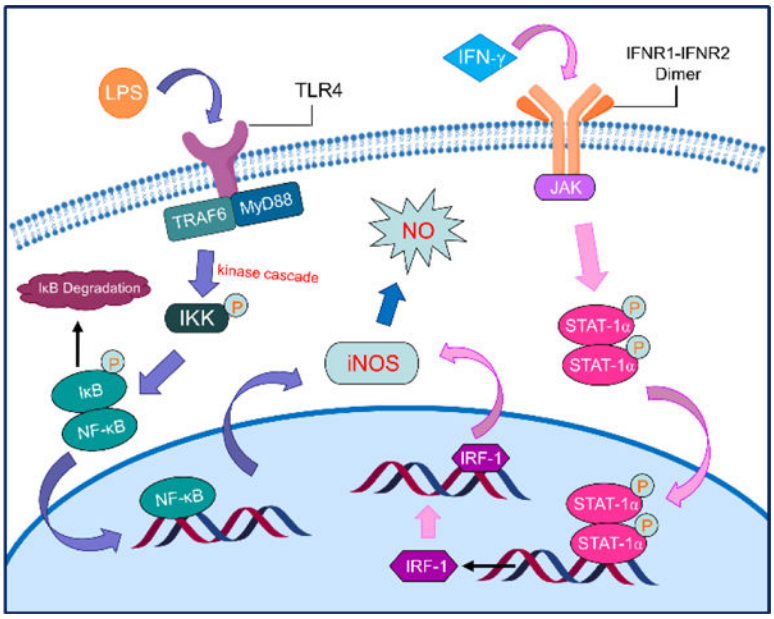


**FIGURE 4.**

Different signaling pathways triggered by different iNOS inducers that regulate iNOS expression (left: activation of NF-κB pathway; right: activation of JAK/STAT-1α pathway).

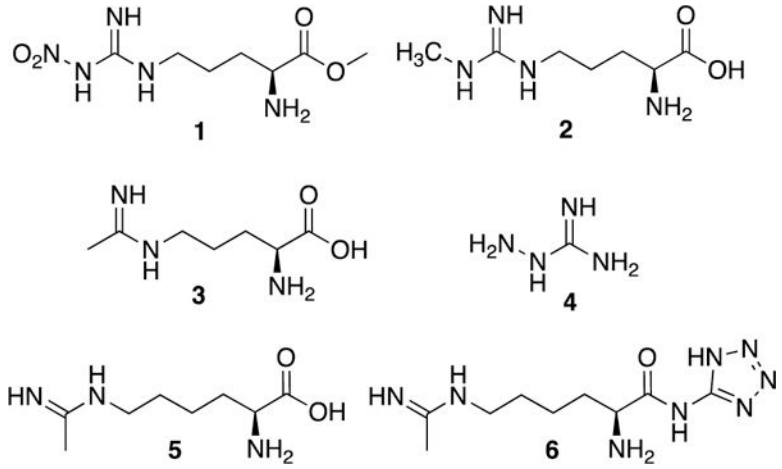


**FIGURE 5.**
Arginine and amino acid amidine derivatives.

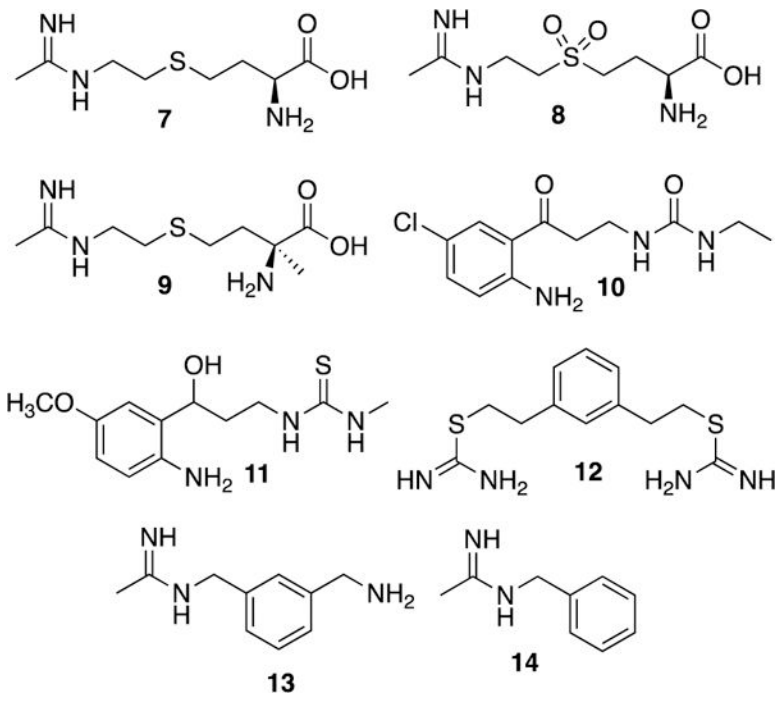


**FIGURE 6.**

Aliphatic and aromatic amidines. When stereochemistry is unknown, a solid line is used (**11**).

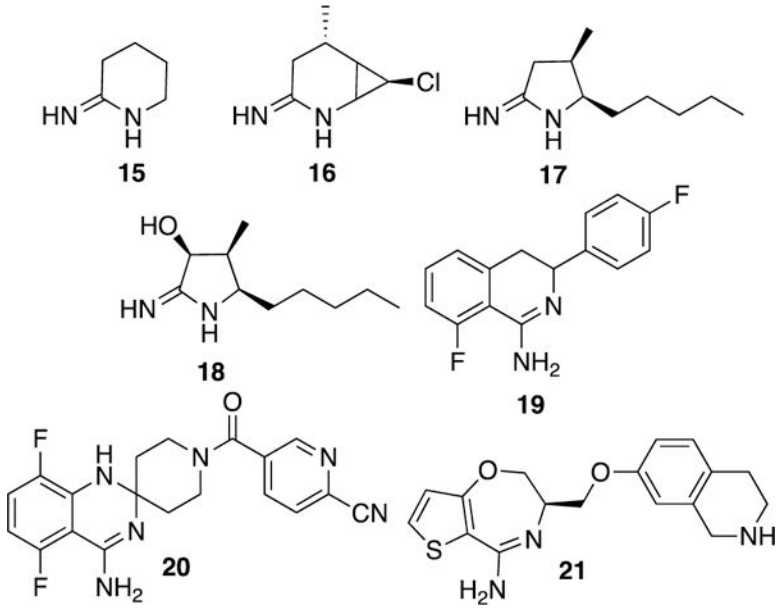


**FIGURE 7.**

Cyclic amidine derivatives. When stereochemistry is unknown, a solid line is used (**19**).

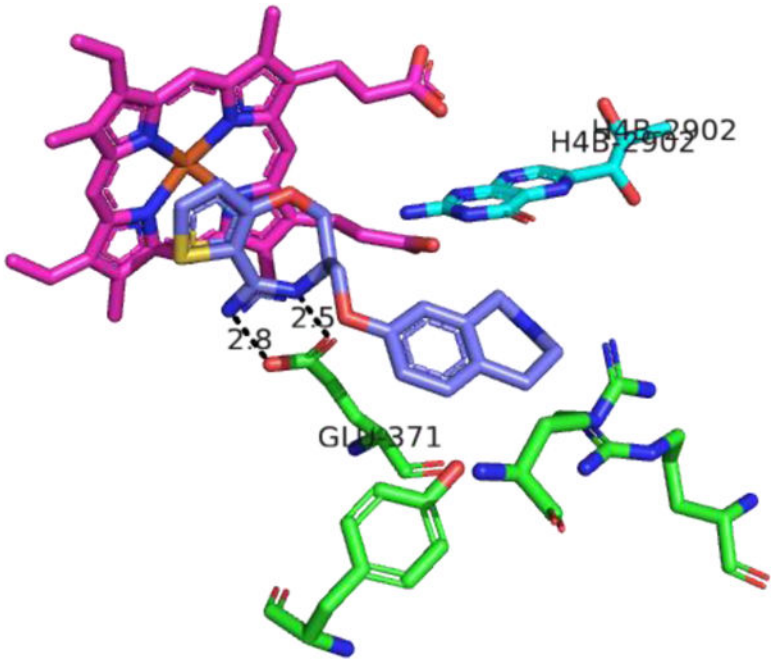


**FIGURE 8.**

X-ray crystal structure of compound **21** (purple) bound in the murine iNOS active site (PDB ID: 3EBF[166]. Heme is depicted in magenta, $H_4B$ in cyan, and amino acid residues of the enzyme in green. Distances are measured in Ångstroms. Figure prepared using PyMol (www.pymol.org).

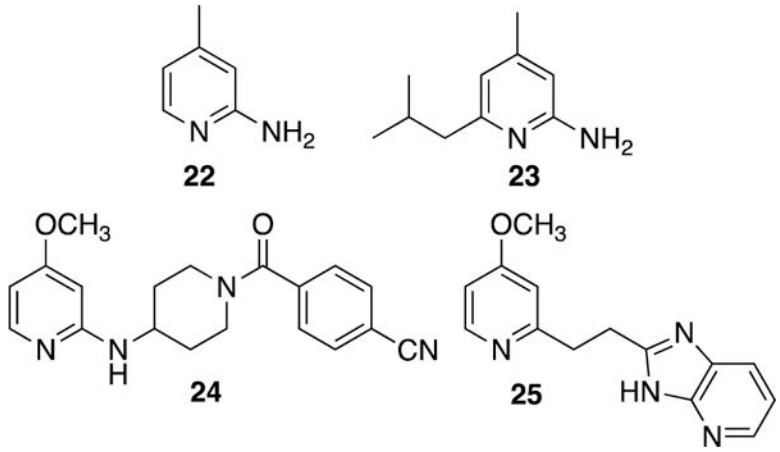


**FIGURE 9.**
Pyridine and aminopyridine derivatives.

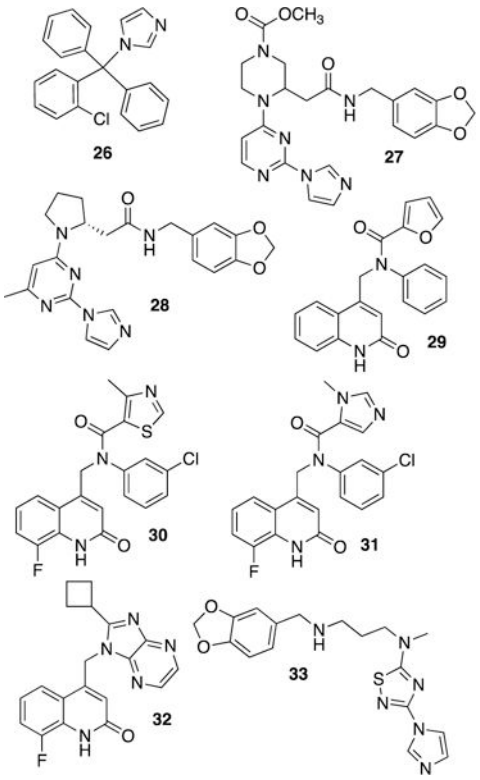


**FIGURE 10.**
Various iNOS dimerization inhibitors. When stereochemistry is unknown, a solid line is used (**27**).

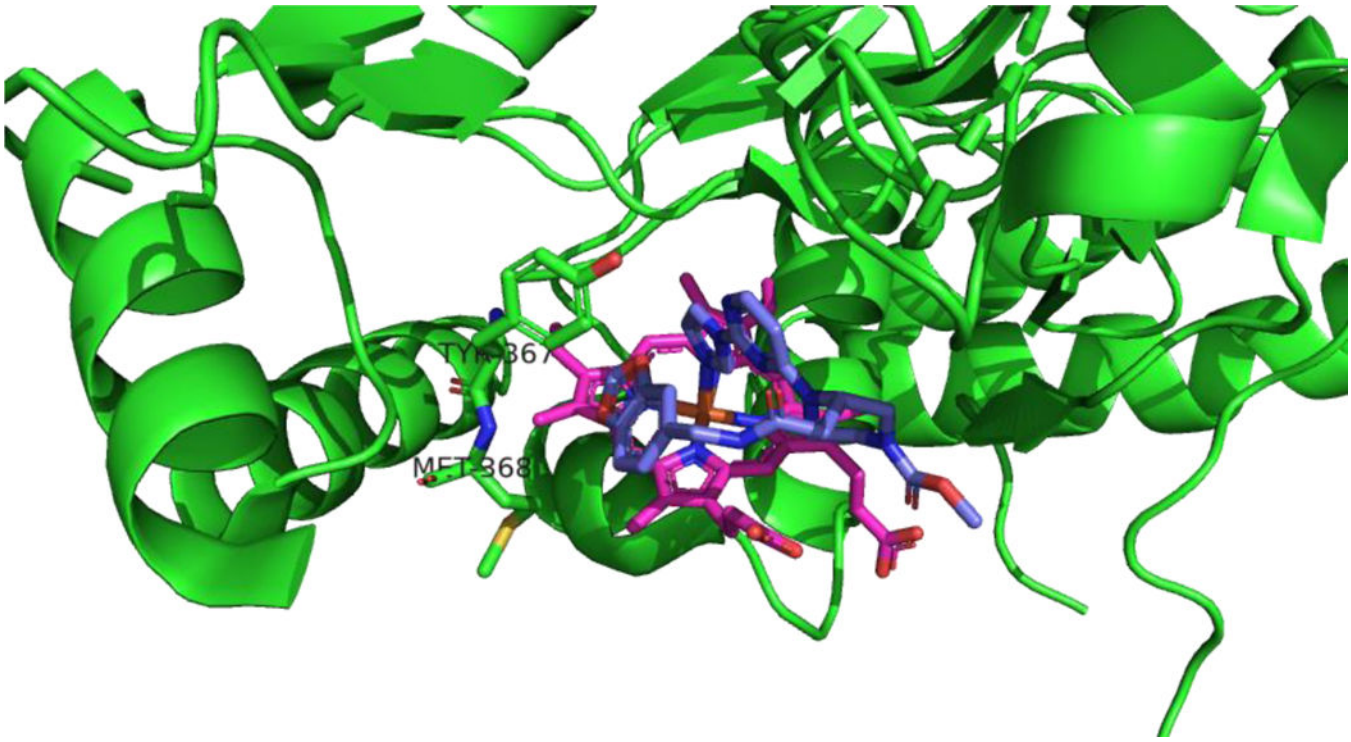


**FIGURE 11.**

X-ray crystal structure of **27** (purple) bound to iNOS monomer (with N-terminal 114 residues truncated). (PDB ID: 1DD7)[175]. Note the coordination of the heme (magenta) iron by the inhibitors's imidazole. The helix containing the catalytic Glu371 is disordered. Figure prepared using PyMol (www.pymol.org).

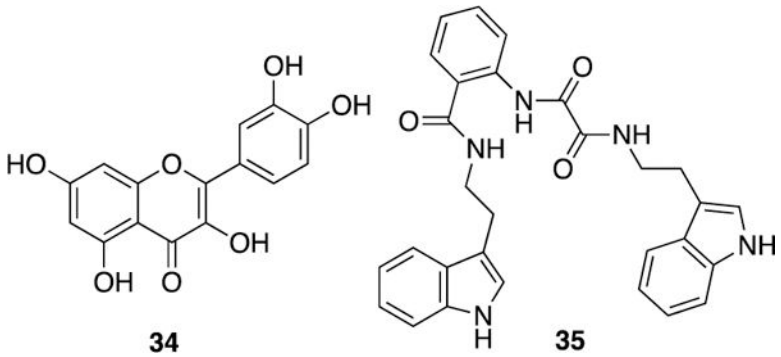


**FIGURE 12.**
Neuroprotective iNOS inhibitors.

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**Table 1.**

Summary of NOS inhibition for relevant compounds

| Compound | Inhibition (μM) | | | Reference | Notes |
|---|---|---|---|---|---|
| | iNOS | eNOS | nNOS | | |
| **1** | 1.4 (human, $K_i$) | 0.06 (human, $K_i$) | 0.09 (human, $K_i$) | 123 | For the acid form, L-NAME is only a weak inhibitor |
| **2** | 2.6 (human, $K_i$) | 0.7 (human, $K_i$) | 1.7 (human, $K_i$) | 123 | Values for competitive inhibition |
| **3** | 2.2 (murine, $IC_{50}$)<br>3.9 (murine, $K_i$) | 3.9 (bovine, $K_i$) | 3.9 (rat, $IC_{50}$)<br>1.7 (rat, $K_i$) | 123, 128 | Values for competitive inhibition; also a mechanism-based inactivator |
| **4** | 54±58 (murine, $IC_{50}$) | Not reported; in vivo results suggest selectivity | 160 (rat, $IC_{50}$) | 131,132 | Little selectivity later reported |
| **5** | 3.3 (murine, $IC_{50}$)<br>0.9 (human, $K_i$) | 16 (human, $K_i$) | 92 (rat, $IC_{50}$)<br>10 (human, $K_i$) | 128, 123 | Values for competitive inhibition |
| **6** | >1850 (human, $IC_{50}$) | 2420 (human, $IC_{50}$) | >1850 (human, $IC_{50}$) | 138 | Is a prodrug; hydrolyzes to **5**, little inhibitory activity |
| **7** | 2.19±0.23 (human, $IC_{50}$)<br><40 nM (human, $K_d$) | 554±61 (human, $IC_{50}$) | 177±44 (human, $IC_{50}$) | 7 | Time and NADPH-dependent competitive inhibitors |
| **8** | 9.03±0.85 (human, $IC_{50}$)<br><90 nM (human, $K_d$) | >1000 (human, $IC_{50}$) | 719±179 (human, $IC_{50}$) | 7 | Time and NADPH-dependent competitive inhibitors |
| **9** | 2.9 (human, $IC_{50}$) | Exact value not reported; around 25-fold weaker | Exact value not reported; around 5-fold weaker | 144 | Time-dependent, irreversible inhibitor |
| **10** | 100 (species not specified, $IC_{50}$) | Exact value not reported; in vivo assays suggest no eNOS inhibition | Exact value not reported; selectivity is reported to be 8-fold | 149 | |
| **11** | 130 (species not specified, $IC_{50}$) | Exact value not reported; in vivo assays suggest no eNOS inhibition | 180 (species not specified, $IC_{50}$) | 148 | Molecular modeling suggests competitive inhibition. |
| **12** | 0.047 (human, $K_i$) | 9.0 (human, $K_i$) | 0.25 (human, $K_i$) | 150 | Proposed to be a competitive inhibitor |
| **13** | 0.007 (human, $K_d$) | 50 (human, $K_i$) | 2.0 (human, $K_i$) | 151 | Originally proposed to be irreversible or a very slow reversible inhibitor |
| **14** | 0.20±0.03 (presumably human, $IC_{50}$) | 350±70 (presumably human, $IC_{50}$) | Not reported | 154 | Competitive inhibitor |
| **15** | 1.0 (human, $IC_{50}$) | 4.7 (human, $IC_{50}$) | 1.1 (human, $IC_{50}$) | 155 | Competitive inhibitor |
| **16** | 0.0019 (human, $K_i$) | 0.018 (human, $K_i$) | 0.0037 (human, $K_i$) | 156, 157 | Competitive inhibitor like **15**. |
| **17** | 0.25 (human, $IC_{50}$) | 226 (human, $IC_{50}$) | 3.2 (human, $IC_{50}$) | 160 | |
| **18** | 0.78 (human, $IC_{50}$) | 1980 (human, $IC_{50}$) | 27 (human, $IC_{50}$) | 161 | |

| Compound | Inhibition (μM) | | | Reference | Notes |
|---|---|---|---|---|---|
| | iNOS | eNOS | nNOS | | |
| **19** | 0.16 (human, $IC_{50}$) | No significant effect at 100 μM (human) | 16 (rat, $IC_{50}$) | 162 | |
| **20** | 0.037 (human, $IC_{50}$) | >100 (human, $IC_{50}$) | 1 (human, $IC_{50}$) | 163 | Competitive inhibitors. |
| **21** | 0.4 (human, $IC_{50}$) | 50 (human, $IC_{50}$) | 1 (human, $IC_{50}$) | 166 | X-ray crystallography indicates competitive inhibitors. |
| **22** | 0.17 (human, $IC_{50}$) | 0.072 (human, $IC_{50}$) | 0.074 (human, $IC_{50}$) | 167 | |
| **23** | 0.028 (human, $IC_{50}$) | 0.15 (human, $IC_{50}$) | 0.10 (human, $IC_{50}$) | 167 | |
| **24** | 0.071 (human, $IC_{50}$) | >100 (human, $IC_{50}$) | 6.6 (human, $IC_{50}$) | 169 | |
| **25** | 0.086 (human, $IC_{50}$) | 162 (human, $IC_{50}$) | 17 (human, $IC_{50}$) | 170, 171 | Competitive, time-dependent, irreversible inhibitor. |
| **26** | 7.3±1 (possibly murine, spectral binding constant for iNOS monomer) | Not reported | Not reported | 174 | Dimerization inhibitor |
| **27** | 0.028 (human, $IC_{50}$, cell lysate)<br>0.0022 (human, $K_i$, purified iNOS monomer) | 32 (human, $IC_{50}$, cell lysate) | 0.140 (human, $IC_{50}$, cell lysate) | 175 | Dimerization inhibitor |
| **28** | 0.00029 (human, $IC_{50}$, A172 cell lysate) | Selectivity reported to be >5000-fold (vaccinia-transfected cell lysate | Selectivity reported to be 62-fold (vaccinia-transfected cell lysate | 178 | Different selectivity values depending on whether vaccinia transfection or tetracycline induction is used |
| **29** | 1.3 (human, $EC_{50,}$ cell-based)<br>46 (murine, $EC_{50,}$ cell-based) | >100 (human, $EC_{50,}$ cell-based) | >10 (human, $EC_{50,}$ cell-based) | 179 | Dimerization inhibitor |
| **30** | 0.011 (human, $EC_{50,}$ cell-based)<br>0.12 (murine, $EC_{50,}$ cell-based) | 26 (human, $EC_{50,}$ cell-based) | 2.3 (human, $EC_{50,}$ cell-based) | 179 | Dimerization inhibitor |
| **31** | 0.009 (human, $EC_{50,}$ cell-based)<br>0.034 (murine, $EC_{50,}$ cell-based) | 6.1 (human, $EC_{50,}$ cell-based) | 0.36 (human, $EC_{50,}$ cell-based) | 179 | Dimerization inhibitor |
| **32** | 0.091 (human, $EC_{50,}$ cell-based) | 16.5 (human, $EC_{50,}$ cell-based) | 0.30 (human, $EC_{50,}$ cell-based) | 181 | Dimerization inhibitor |
| **33** | ~0.003 (species not specified, $IC_{50}$) | >30 (species not specified, $IC_{50}$) | ~2.4 (species not specified, $IC_{50}$) | 182 | Dimerization inhibitor |
| **34** | Not reported, but lowers iNOS expression in PC12 cell cultures | Not reported | Not reported | 56 | Exact mechanism of action unknown; possibly multiple targets |
| **35** | Inhibits NO production in LPS-stimulated macrophages at 20 μM | Not reported | Not reported | 200 | Exact mechanism of action unknown; proposed to be a competitive inhibitor |