Biochemical Pharmacology, Vol. 51, pp. 383–394, 1996.

ISSN 0006-2952/96/$15.00 + 0.00
SSDI 0006-2952(95)02099-3

COMMENTARY

Selective Pharmacological Inhibition of Distinct Nitric Oxide Synthase Isoforms

*Garry J. Southan and Csaba Szabó**

DIVISION OF CRITICAL CARE, CHILDREN'S HOSPITAL MEDICAL CENTER, CINCINNATI, OH 45229, U.S.A.

ABSTRACT. Nitric oxide (NO) is produced in physiological and pathophysiological conditions by three distinct isoforms of NO synthase (NOS): endothelial NOS (ecNOS), inducible NOS (iNOS), and brain NOS (bNOS). Selective inhibition of iNOS may be beneficial in various forms of shock and inflammation, whereas inhibition of bNOS may protect against neuroinjury. This article surveys the enzymatic mechanism of NO production, lists the strategies and pharmacological tools for selective inhibition of distinct NOS isoforms, and considers the side-effects of the various approaches. Selective inhibition of NOS isoforms is achieved by: (a) targeting the differential co-factor (calmodulin or tetrahydrobiopterin) requirement of various NOS isoforms of NOS; (b) targeting the differential substrate requirements of cells expressing various isoforms of NOS (L-arginine uptake blockers or arginase); (c) the use of pharmacological agents that are selectively taken up by cells expressing various isoforms of NOS (7-nitroindazole); or (d) developing pharmacological NOS inhibitors with isoform specificity. The amino acid-based NOS inhibitor, N^G-nitro-L-arginine, shows a preference for ecNOS and bNOS over iNOS, whereas L-N^6-(1-iminoethyl)lysine is selective for iNOS over bNOS. Certain non-amino acid-based small molecules, such as aminoguanidine and certain S-alkylated isothioureas, also express selectivity towards iNOS and have anti-inflammatory and anti-shock properties. 7-Nitroindazole, a bNOS-selective inhibitor, protects in central nervous system injury. Clearly, there are a number of distinct approaches that are worthy of further research efforts in order to achieve even more selective targeting of various NOS isoforms. BIOCHEM PHARMACOL 51;4:383–394, 1996.

KEY WORDS. nitric oxide; endothelium; macrophage; selectivity; L-arginine; N^G-nitro-L-arginine; 7-nitroindazole; isothioureas; amidines; aminoguanidine; inflammation; shock; vascular effects

ACTIONS AND BIOLOGICAL ROLES OF NO†

Physiological and Pathophysiological Roles of NO

NO is a small membrane-permeable gas that serves as a mediator of many physiological events. It is produced by the oxidation of L-arginine by a family of isoenzymes—NOS [1–3]. NO produced by the ecNOS is involved in the regulation of blood pressure, organ blood flow distribution, and the inhibition of the adhesion and activation of platelets and polymorphonuclear granulocytes [4]. Inhibition of the actions of ecNOS by N^G-substituted analogues of L-arginine, such as L-NMA or L-NA, causes vasoconstriction, increases blood pressure, and reduces blood flow to organs. Dysfunction of NO formation in the vascular endothelium has been implicated in the pathophysiology of a number of vascular and non-vascular diseases [4, 5].

The NOS isoform present in the central and peripheral nervous system (bNOS) is another constitutive, calcium/calmodulin-dependent enzyme [6]. NO derived from bNOS has important physiological roles as a neurotransmitter and as a potential mediator of the metabolism/blood flow coupling in the brain. Enhanced formation of NO (e.g. following activation of *N*-methyl-D-aspartate receptors or induction of iNOS in the microglia) plays a role in neuroinjury [7, 8], suggesting that inhibition of bNOS may be of therapeutic benefit in central nervous system injury.

A distinct isoform of NOS (iNOS) can be induced by pro-inflammatory agents such as endotoxin (bacterial lipopolysaccharide, LPS), interleukin-1β, tumor necrosis factor-α and interferon-γ in a variety of cells, including macrophages and smooth muscle cells [9]. Enhanced formation of NO following the induction of iNOS has been implicated in the pathogenesis of a number of conditions, including various forms of circulatory shock [10–13] and inflammation [14, 15]. The pathophysiological importance of an enhanced formation of NO by iNOS suggests that inhibitors of iNOS have therapeutic potential, particularly those that do not interfere with the protective and physiological roles of ecNOS.

NOS: Catalytic Activity and Sites of Potential Inhibition

NOS produces NO by the oxidation of one of the guanidino nitrogens of arginine (or certain other substrates). The process

* Corresponding author: Dr. Csaba Szabó, Division of Critical Care, Children's Hospital Medical Center, 3333 Burnet Ave., Cincinnati, OH 45229. Tel. (513) 559-4259; FAX (513) 559-4267.

† *Abbreviations*: BH_4, tetrahydrobiopterin; L-NA, N^G-nitro-L-arginine; L-NAME, N^G-nitro-L-arginine methyl ester; L-NMA, N^G-methyl-L-arginine; NO, nitric oxide; NOS, nitric oxide synthase(s); bNOS, brain nitric oxide synthase isoform; ecNOS, endothelial constitutive nitric oxide synthase isoform; iNOS, inducible nitric oxide synthase isoform; and ITU, isothiourea.

involves the oxidation of NADPH and the reduction of molecular oxygen. The transformation occurs at a catalytic site adjacent to a specific binding site for L-arginine. The catalytic site includes an iron-containing heme group, iron protoporphyrin IX, in which the ferric iron is coordinated to the tetrahentate porphyrin in a planar arrangement. The iron is also coordinated to the thiol of a cysteine residue (thought to be cysteine 184 in ecNOS and cysteine 415 in bNOS [16, 17]), thus linking the heme to the rest of the protein. The sixth coordination site is available for the binding and activation of molecular oxygen during the stepwise oxidation of L-arginine [2, 3].

Flavin-adenine dinucleotide and flavin mononucleotide are both co-factors and, by analogy with their function in P450 reductases, as thought to be involved in the transfer of electrons from NADPH to the catalytic center. It has been suggested that the flavoprotein inhibitor, diphenyleneiodonium, exerts its inhibition of NOS activity by its irreversible effects on the FAD binding site [18].

Cloning of the isoenzymes has revealed that NOS subunits are divided into a reductase domain and an oxygenase domain, the latter containing the catalytic center. These domains are linked by a sequence that can bind calmodulin. The binding of calmodulin appears to be essential for NOS activity and is thought to control the electron transfer from the flavins to the heme, possibly by causing a reorientation of the reductase and oxygenase domains to allow electron transfer between them. The binding of calmodulin to native (inactive) constitutive isoforms of NOS is promoted by calcium ions. Consequently, the activities of bNOS and ecNOS are regulated by the concentrations of intracellular calcium. In contrast, calmodulin is tightly bound as a prosthetic group to the inducible NOS holding it in an activated state that is independent of intracellular calcium.

The oxygen domain of NOS has a highly conserved 320-amino acid sequence that is likely to contain binding sites for BH_4 as well as heme and L-arginine, which are likely to be in close proximity [19]. The function of BH_4 is unclear, but it may act as an allosteric effector or stabilizer of the active structure of NOS (i.e. assembling dimeric forms of NOS in the case of bNOS and iNOS) [2, 3].

The synthesis of NO by NOS involves the binding of L-arginine so that the guanidino group is adjacent to the ferric iron of the heme prosthetic group. The iron is reduced by transfer of an electron from NADPH and binds molecular oxygen, which is then cleaved, with one oxygen atom released as water and the other incorporated onto one of the terminal guanidino nitrogens of arginine to yield hydroxyarginine. Subsequent activation of a further molecule of oxygen facilitates the further oxidation of hydroxyarginine to produce water, NO, and citrulline. The chemistry of the process remains to be clarified (see Refs. 2, 3, and 20–22 for details and discussion), but it is evident that the guanidino group of the substrate must be in close proximity to the (normally vacant) sixth coordination site of the heme iron where molecular oxygen binds and becomes activated for subsequent reaction with substrate. Coordination of a sixth ligand perturbs the arrangement of the iron within the porphyrin and results in a spectral shift that is dependent on the ligand incorporated [2, 3, 22]. Occupation of this site by ligands other than oxygen would prevent the oxidation of L-arginine and is probably involved in the mode of action of NOS inhibition by small molecules such as carbon monoxide, NO (although thiols and amines are other potential sites), and many nitrogen-containing species. This mechanism on its own is unlikely to provide any discrimination between various NOS isoforms.

NOS isoforms have narrow specificity with respect to the substrates utilized to produce NO. Besides the natural substrate, L-arginine, the only other compounds reported or proposed as substrates for NOS are Nα-benzoyl-L-arginine, L-homoarginine, ϵ-guanidinocapronic acid, canavanine, L-N^G-methoxyarginine and L-N^G-hydroxyarginine [23, 24]. L-NMA, a widely used inhibitor of NOS (see below), is also capable of NOS-dependent generation of NO [25], although the proposed mechanisms suggest either L-arginine or hydroxyarginine as intermediates. The D-isomers of the above-listed compounds are not substrates, nor are they inhibitors. Interestingly, in the absence of substrate, NOS has been shown to produce superoxide anion and hydrogen peroxide [26].

iNOS utilizes large amounts of L-arginine, and this may result in depletion of intracellular L-arginine pools and/or an increased uptake of L-arginine from extracellular sources. Induction of iNOS is also associated with the induction of arginosuccinate synthetase, which, by stimulating the "re-cycling" of L-citrulline to L-arginine, may support iNOS with its substrate from endogenous sources [27]. In some cells, the same stimuli that induce iNOS also enhance the membrane transport system for L-arginine [28]; prolonged activation of iNOS may require the replacement of intracellular L-arginine from extracellular sources [29]. Some of the NOS inhibitors (e.g. L-NMA) also inhibit the transporter system, whereas others (e.g. L-NA) do not affect it [30]. The transport systems can also be inhibited in a competitive fashion by cationic amino acids such as L-lysine and L-ornithine [29]. The extensive extracellular L-arginine requirement of cells expressing iNOS may offer a target for pharmacological intervention: extracellular arginase [31] or inhibition of L-arginine transport with specific agents such as the tetravalent guanylhydrazone CNI-1493 [32] has been shown to provide therapeutic benefit in experimental models of septic shock.

Differential co-factor requirements of various NOS isoforms offer a way for selectively targeting NOS isoforms. For instance, the calmodulin-dependent NOS isoforms (ecNOS and bNOS), as with most calmodulin-dependent enzymes, can be inhibited by calmodulin inhibitors, such as calmidazolium [33]. iNOS, which contains calmodulin in a tightly bound form, is not inhibited by these agents. While antagonism of calmodulin can provide a basis for selectivity, the existence of many other calmodulin-dependent biological processes makes the usefulness of this approach questionable.

BH_4 has been suggested to be a critical co-factor for iNOS. Inhibition of BH_4 production by 2,4-diamino-6-hydroxypyrimidine, a selective inhibitor of GTP cyclohydrolase I, the rate-limiting enzyme in the production of BH_4, inhibits NO production by iNOS [34, 35]. In addition, it has been shown that *N*-acetylserotonin, phenprocoumon and dicumarol, which inhibit the synthesis of BH_4 by blocking sepiapterin

reductase, also reduce cytokine-induced NO production [35, 36]. These data suggest that inhibitors of BH_4 biosynthesis may be useful in preventing NO formation by iNOS, especially since phenprocoumon and dicumarol, while inhibiting iNOS activity, do not block NO production by ecNOS [36]. In apparent agreement with the *in vitro* results, *N*-acetylserotonin pretreatment has been shown to reduce endotoxin-induced hypotension in the rat [37]. However, recent data indicate that *N*-acetylserotonin also inhibits the *induction* of iNOS [38], thus making the prior experimental results based on pharmacological actions of *N*-acetylserotonin difficult to interpret. The use of BH_4 synthesis inhibitors may also be hampered by the fact that BH_4 is a co-factor for tyrosine hydroxylase and tryptophan hydroxylase (among other enzymes) and so inhibition of BH_4 production may lead to reduced biosynthesis of dopamine, epinephrine, norepinephrine, and serotonin [39–41].

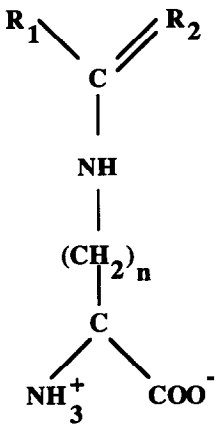
It has been suggested that modulation of NOS activity can be achieved by selective phosphorylation of NOS as illustrated by the neuroprotective effect of the immunosuppressant drug FK506. This drug appears to inactivate iNOS by inhibiting its dephosphorylation [42]. Although similar mechanisms have not been reported for ecNOS or iNOS, the isoform-selectivity of the above process needs to be further explored.

STRUCTURE AND BIOLOGICAL ACTIONS OF L-ARGININE-BASED INHIBITORS OF NO SYNTHASES

Many analogues of L-arginine are inhibitors of NOS, in particular those substituted at one or both of the terminal guanidino (amidino or ω–) nitrogens [22, 43] (Fig. 1). On the whole, the L-isomers are far more effective inhibitors of NOS than the corresponding D-isomers, which are not generally inhibitors, although one exception is known: both D-NA and L-NA inhibit endothelium-dependent relaxations in rat aortic rings [44]. Compounds substituted on the propylene chain are generally neither inhibitors nor substrates. Similarly, removal of the α-amino or α-carboxyl groups reduces recognition by the arginine binding site. For instance, 5-guanidino pentanoic acid (argininic acid) and 4-aminobutylguanidine (agmatine) are neither substrates nor noteworthy inhibitors of either bNOS or iNOS [24, 25].

Even esterification of the carboxyl group can reduce inhibitory potency markedly. L-NAME, often regarded as equivalent to L-NA, is, in fact, over an order of magnitude less potent towards iNOS than L-NA [45]. In principle, the fact that esterase activity is needed for L-NAME to fully exhibit its inhibitory action may offer the opportunity for selective targeting of NOS in various tissues (with specific esterase activities) using appropriate esters of NOS inhibitors.

All of the NOS isoforms can be inhibited (to a variable degree) with N^G-substituted L-arginine analogues, e.g. L-NMA. Some analogues of L-arginine do exhibit some isoform selectivity, mostly towards the constitutive isoforms. N^G-Cyclopropyl-L-arginine shows a preference for bNOS over iNOS *in vitro* [46], whereas L-NA and L-NAME (after hydrolysis) show selectivity toward ecNOS. L-NMA and N^G-amino-L-arginine show no marked preference for either isoform [46–50]. The mechanisms of inhibition of NOS activity by these compounds vary, but all involve occupation of the substrate bind-



	R_1	R_2	n
L-arginine	NH_2	NH	3
L-NMA	NH_2	NCH_3	3
L-NA	NH_2	NNO_2	3
L-NAME*	NH_2	NNO_2	3
L-amino-arginine	NH_2	NNH_2	3
L-cyclopropyl-arginine	NH_2	NC_3H_5	3
L-allyl-arginine	NH_2	NCH_2CH_3	3
L-iminoethyl-ornithine	CH_3	NH	3
L-N6-iminoethyl-lysine	CH_3	NH	4
L-homoarginine	NH_2	NH	4
L-citrulline	NH_2	O	3
L-thiocitrulline	NH_2	S	3
S-methyl-thiocitrulline	SCH	NH	3
S-ethyl-thiocitrulline	SCH_2CH_3	NH	3
L-hydroxyarginine	NH_2	NOH	3

*** in L-NAME, the acid function (- COO^-) becomes (- CO - O CH_3)**

FIG. 1. Structures of L-arginine, L-citrulline, L-hydroxyarginine, L-homoarginine and various amino acid-based NOS inhibitors.

ing site. Simplistically, this excludes L-arginine and hence prevents its metabolism to NO. Indeed, many L-arginine-based inhibitors (cyclopropyl-L-arginine, L-NA) are competitive inhibitors of NOS in that their effects can be reversed by L-arginine. However, their influence on electron transfer and reduction of the heme iron in the NOS molecule may differ. L-NAME appears to inhibit NADPH consumption by interrupting electron flux immediately prior to reduction of the heme iron, whereas L-NMA does not influence the reduction of the heme iron, but uncouples NADPH oxidation from catalytic activity [51]. Some compounds such, as *N*-iminoethyl-L-ornithine [52], show inhibition that cannot be reversed by L-arginine (or other factors). Such irreversible inhibition can be associated with alterations of inhibitor binding to reduce the rate of dissociation of the inhibitor/NOS complex or chemical modification of the inhibitor and/or enzyme. For instance, exposure of iNOS to L-NMA results in a time-dependent irreversible inactivation of the enzyme, which is preceded by an NADPH-dependent hydroxylation of the inhibitor to *N*-hydroxy-*N*-methyl-L-arginine [20]. In addition, L-NMA may cause a significant loss of heme [53] from the enzyme. Similarly, the irreversible component of the inhibition of NOS by N^G-allyl-L-arginine probably involves generation of an intermediate capable of covalent binding to the enzyme. Interestingly, irreversible inhibition of bNOS by L-NA has also been reported [54], in contrast to the reversible nature of its inhibition of iNOS. This illustrates differences in the binding characteristics of NOS isoforms that offer a potential rational basis for drug development.

Dimethyl-arginines, such as symmetric (*N,N'*-dimethylarginine) and asymmetric dimethylarginine (*N,N*-dimethylarginine) are endogenously produced inhibitors of NOS, but do not show any isoform selectivity [23]. However, the plasma levels of these compounds are known to increase in chronic renal failure [55, 56] and in hypercholesterolemia [57].

Despite their lack of marked isoform selectivity and certain non-specific effects (see below), L-arginine-based inhibitors have been used widely in the last 5 years in order to elucidate the pathophysiological importance of NO and have demonstrated beneficial effects in various forms of shock, inflammation, and central nervous system disorders [1, 2, 5, 7, 11–14, 42, 43, 58–66]. It appears that in shock and inflammation. NO derived from iNOS is responsible for most of the pathophysiological actions, whereas, in many cases, ecNOS has beneficial and protective roles and so its inhibition is not advantageous. Excessive inhibition of ecNOS may mask the beneficial effects of inhibiting iNOS, as in endotoxin-induced intestinal injury [64] or liver failure [65–67]. It follows that L-arginine-based inhibitors of NOS may not be ideal when targeting a particular isoform of NOS (especially iNOS) due to their limited selectivity and the relative low potency. The ready *in vitro* reversibility of their effects by L-arginine, together with the fact that millimolar concentrations of L-arginine are present in the plasma, may explain the need for large doses of these agents to achieve biological effects.

The requirement for high doses of inhibitors not only exaggerates their effects on other isoforms of NOS, but also reveals an increasing number of non-specific actions of L-arginine-based inhibitors. Some of these effects impinge on the arginine-NO system: L-NMA, but not L-NA, affects arginine transport into the cell (a rate-limiting step in NO production by iNOS) [30]. L-NA has been shown to inhibit arginase activity (which catalyzes the conversion of L-arginine to L-ornithine), while L-NMA, L-NAME and aminoguanidine do not [68]. These effects are properties of a particular inhibitor, rather then general properties of all L-arginine-based inhibitors. L-NMA can act as a substrate for NO production by NOS in the rat aorta and rabbit pulmonary artery [25]. In isolated rat aortic rings, L-NMA (as well as L-NA) inhibits the "agonist-induced" endothelium-dependent (ecNOS-mediated) relaxations elicited by acetylcholine, but (unlike L-NA) does not augment the phenylephrine-induced contractions in the same vessel [69], even though this augmentation is generally considered as an indicator of "basal" release of NO by ecNOS. Although there is some speculation about different types of endothelial NOS or changes induced in the enzyme that allow utilization of L-NMA as a *substrate* [25, 69], there is no definitive explanation for these findings. Another commonly used agent, L-NAME, requires hydrolysis to become effective against iNOS. This probably occurs rapidly *in vivo*, but can lead to possible complications *in vitro*, where short incubation times, leading to partial hydrolysis, are used. Similar considerations apply when using other esters and amides of arginine analogues (e.g. L-NA-*p*-nitroanilide; [45]). L-NAME and other esters of L-arginine, but not other NOS inhibitors tested (such as L-NMA), have been reported to act as muscarinic receptor antagonists [70].

More general, non-NOS-specific properties of arginine analogues may include their inhibition of the activity of iron-containing enzymes such as catalase [71] and effects on iron-containing systems, in general, as illustrated by the attenuation by NOS inhibitors of the inhibitory effect of ferrous iron on the reduction of cytochome *c* [72].

In addition to these non-specific effects of L-arginine-based NOS inhibitors *in vitro*, unexpected side-effects of NOS inhibitors can also be observed *in vivo* in some cases. For instance, L-aminoarginine has been reported to cause epileptiform seizures in dogs [73], an effect that may be related either to effects of NO blockade in the central nervous system, or, alternatively, to a non-specific effect of the drug itself.

OTHER AMINO ACID-BASED INHIBITORS OF NO SYNTHASES

Recent work has demonstrated that derivatives of L-citrulline and L-lysine can be potent inhibitors of NOS [74–76], some of them with isoform selectivity. L-Thiocitrulline (Fig. 1) is an extremely potent inhibitor of all NOS isoforms, which binds to the heme moiety of the NOS molecule, thereby reducing its redox potential [51]; its effects are reversed dose dependently by L-arginine [77]. L-Thiocitrulline has no particular isoform selectivity [77] and elicits similar potent pressor effects in both normal and endotoxemic rats [77], suggesting that it can be only of limited therapeutic use for targeting iNOS. In contrast, *S*-ethyl- and *S*-methyl-thiocitrulline (both of which can be

considered as isothioureas, see below and Fig. 1) are more potent inhibitors of bNOS than of ecNOS or iNOS [74], and thus may be of therapeutic potential in various neurodegenerative diseases that are associated with NO overproduction by bNOS. However, the therapeutic usefulness of *S*-ethyl- and *S*-methyl-thiocitrulline may be limited due to its poor uptake into cells [74]. Their effects are arginine-reversible and, like L-NA (but unlike L-NMA), they inhibit the NADPH oxidase activity of bNOS [74].

Recently, the Monsanto Co. has published on the development and some biological actions of the novel NOS inhibitor L-N^6-(1-iminoethyl)lysine, which exhibits a 30-fold selectivity for iNOS over bNOS [76, 78]. Although data regarding the selectivity of the agent on iNOS over ecNOS have not been disclosed, preliminary data indicate that the compound exerts potent anti-inflammatory actions in a rat model of adjuvant arthritis [78].

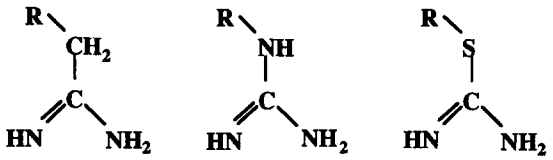
NON-AMINO ACID-BASED INHIBITORS OF NO SYNTHASES

Arginine and its analogues can be regarded as derivatives of guanidine, so that the boundary between an arginine analogue and a non-arginine-based compound is vague and somewhat arbitrary. For the purposes of this review, we will consider compounds without both α-amino- and α-carboxylic acid groups as non-arginine-based compounds as they no longer possess the amino acid group, which has its own requirements for binding to a portion of the substrate binding site of NOS.

Non-arginine-based inhibitors of NOS represent probes to determine the structure of the L-arginine binding site/catalytic site of iNOS without constraints imposed by the specific stereochemistry of the binding of an amino acid group. Hence, they may prove to be useful tools in the design of isoform-specific inhibitors. Furthermore, demonstration of anti-NOS activity may now provide explanation for the mechanism of previously known actions of various biologically active compounds (see below).

Despite a requirement for the conserved stereochemistry of arginine analogues, a variety of compounds that are not amino acids are inhibitors of NOS activity. Examples include imidazoles [79, 80], 7-nitroindazoles [81], guanidines [82], iminobiotin [83], ebselen and derivatives [84], α-guanidinoglutaric acid [85], tannin [86], 3-amino-1,2,4-triazole [87], phencyclidine [88], and methylene blue [89]. Among this large and continually increasing list of inhibitors, only a few groups of compounds, namely aminoguanidines, isothioureas, and certain imidazoles and indazoles, have been reported to exhibit isoform selectivity and will be discussed below.

Guanidines (Fig. 2b) have long been known to cause pressor responses in various animals, and their similarity to L-arginine (a substituted guanidine) led to their investigation as inhibitors of NOS. Guanidine itself is not an inhibitor, but methylguanidine (an endogenous substance) and ethylguanidine do inhibit NOS, although they are weaker than L-NMA (authors' unpublished observations). 1,1-Dimethylguanidine is somewhat more potent against iNOS (induced in insulinoma cells) than methylguanidine, but is still weaker than L-NMA. However, it appears to be equivalent to L-NMA in its effect on ecNOS in mesenteric artery preparations and causes rapid increases in MAP *in vivo*. Arcaine, containing two guanidine groups (1,4 diguanidinobutane), shows moderate inhibition of NOS [24].



a) amidines b) guanidines c) isothioureas

FIG. 2. General structures of amidine derivatives.

Other alkyl-substituted guanidines show weak or negligible effects on NOS. On the other hand, *aminoguanidine* (Fig. 2b, R = $-NH_2$) has received much attention as an inhibitor of NOS due to the early recognition of its selectivity towards iNOS [90], its low acute toxicity, and its potential clinical usefulness. There are now numerous reports on the beneficial effects of aminoguanidine in various experimental models of inflammation and shock. In most *in vitro* systems, aminoguanidine is nearly equipotent with L-NMA in its ability to inhibit NO production by immunostimulated macrophages. On the other hand, aminoguanidine is less potent than L-NMA on ecNOS and bNOS activities in cell homogenates [65, 91] and on ecNOS *in vivo* (as demonstrated by its limited pressor effects in anesthetized rats). However, it has been noted that when aminoguanidine is incubated with cells or tissues containing NOS or with semi-purified NOS, the length of the incubation affects the apparent potency: short incubation times result in a reduced inhibitor action [91, 92]. Such a difference is not found for other inhibitors such as *S*-methyl-isothiourea (see below) or L-NMA. Moreover, in certain experimental systems the selectivity of aminoguanidine towards iNOS appears to be rather limited, for example, when comparing its effects on ecNOS and iNOS from intestinal homogenates [93]. In *in vivo* experiments, doses of aminoguanidine required to elicit a considerable inhibition of iNOS (and thus, elicit beneficial biological effects) are rather high: 15–45 mg/kg, i.v., in anesthetized rats or up to 400 mg/kg/day in mice [91, 94, 95].

Aminoguanidine has been studied extensively in diabetes where it appears to exert NO-dependent and NO-independent beneficial effects. Aminoguanidine prevents the interleukin-1-induced decrease in glucose secretion in islets of Langerhans and reduces the glucose-induced vascular dysfunction in rats [90, 96]. Its effects in inhibiting diabetic vascular dysfunction also appear to be NO-mediated [97], whereas the NO-dependent [61, 62] development of diabetes in a low-dose streptozotocin model may [94] or may not [98] be prevented by aminoguanidine. Aminoguanidine has been shown to restore contractile responses in vascular tissues of animals treated with endotoxin *in vivo* and *in vitro* (an iNOS-dependent phenomenon) [90–92, 99]. In addition, aminoguanidine improves the survival rate in mice treated with endo-

toxin [91] to an extent comparable with the improvement in survival seen in the iNOS knock-out transgenic mice treated with endotoxin [66].

Other effects of aminoguanidine include anti-inflammatory actions: it inhibits the progression of experimental autoimmune encephalomyelitis [95] and the cardiovascular consequences of experimental pancreatitis [100], as well as prevention of the endotoxin-induced acute pulmonary extravasation [101]. All these biological effects of aminoguanidine have been tentatively linked to its inhibitory effect on iNOS. The clear evaluation of the *in vivo* effects of aminoguanidine as an inhibitor of iNOS, however, is hampered by the fact that it has numerous other effects. These include inhibition of histamine metabolism [102], inhibition of polyamine catabolism [103], and inhibition of catalase [104] and other copper- or iron-containing enzymes. Other beneficial, but NO-*in*dependent, effects of aminoguanidine are its reduction of end product glycosylation in diabetes and its inhibitory effect on oxidative modification of low density lipoprotein [105]. These effects may be consequences of the reactivity of aminoguanidine in providing an alternative substrate for glycosylation and, in the latter case, by reacting with aldehydes formed during lipid peroxidation. Aminoguanidine also potentiates the effect on angiotensin-induced prostacyclin production in rat lungs, an effect not seen with L-NA [106]. Apparently, aminoguanidine can be tolerated in high doses in experimental animals, although chronic administration may cause side-effects, as its hydrolysis can give rise to semicarbazide and hydrazine, both toxic products.

Certain derivatives of aminoguanidine, such as *N,N'*-diaminoguanidine, although exhibiting lower potency than aminoguanidine, appear to be selective for iNOS [82]. The mechanism of inhibition of NOS by aminoguanidines (and possibly guanidines) if thought to be due to their binding as ligands to the heme iron at the catalytic site as aminoguanidine deactivates other iron- or copper-containing enzymes in this manner. Competitive binding with L-arginine is probably involved as L-arginine can attenuate the pressor responses to some guanidines [82]. However, this explanation does not address the apparent selectivity of some potential ligands and not others. Recent data show that the inhibition of NOS by aminoguanidine becomes greater with increasing incubation time [91, 92, 107], indicating that aminoguanidine is a mechanism-based inhibitor [107]. Inactivation of various catalytic functions of NOS by aminoguanidine (NADPH oxidase and citrulline formation but not the cytochrome c reductase activity) is dependent on an active NOS and the appropriate NOS co-factors [107].

Several compounds reported individually to inhibit NOS also contain the guanidino group. Inhibition of NOS activity by α-guanidinoglutaric acid [85] and by 3-amino-1,2,4-triazole [87] has been reported, but only for one isoform each (bNOS and iNOS, respectively). Iminobiotin [83], which is structurally related to mercaptoalkylguanidines (below), shows no discrimination between iNOS and bNOS.

The most potent guanidino inhibitors of iNOS reported to date are *mercaptoalkyl-guanidines*, in particular mercaptoethyl- and mercaptopropyl-guanidines (Fig. 2c, R = —CH_2CH_2SH and —$CH_2CH_2CH_2SH$, respectively) [108]. They are far more effective than L-NMA at inhibiting iNOS in whole cells, but mercaptoethylguanidine is much less effective against ecNOS *in vitro* and causes only slight pressor responses in anesthetized rats [107]. Mercaptoethyl-guanidine (and possibly the isothiourea, 2-aminothiazoline) is probably the active form of aminoethyl-isothiourea (Fig. 2b, R = —$CH_2CH_2NH_2$)*, a recently identified inhibitor of NOS [109, 110]. Thus, the beneficial effects of aminoethyl-isothiourea on iNOS-dependent pathophysiological processes in endotoxemia (reversal of hypotension* and protection against endotoxin-induced liver damage [111]) are likely to be due to mercaptoethyl-guanidine. Delineation of the actions of mercaptoalkyl-guanidines and related compounds are hampered by the ease of their oxidation to the disulfide in aqueous solution, a process that occurs *in vivo* [112]. It is worthy of note that mercaptoalkyl-guanidines, including mercaptoethyl-guanidine, have been described as radioprotective agents [112, 113], in view of the recent report that inhibitors of NOS *in vivo* also protect against exposure to radiation [114]. Substituents on the sulfur, as in *S*-methyl- or *S*-ethyl-mercaptoalkyl-guanidines, appear to reduce the potency of NOS inhibition, but maintain the selectivity towards iNOS as compared with ecNOS (authors' unpublished observations). Such compounds may have the advantage that they do not dimerize. Little is known, as yet, about the nature of the inhibition of NOS by mercaptoalkyl-guanidines, other than it is of a competitive nature. By analogy with other guanidines, binding to the heme of NOS is likely either through the guanidino group or the sulfur. As with other inhibitors that contain sulfur or selenium (e.g. carboxyebselen [84]), there is always the possibility of interaction with the cysteine residue that is normally coordinated to the heme iron.

Guanidines, in general, exhibit numerous pharmacological properties that are independent of their inhibition of NOS, including inhibition of the membrane sodium/potassium ATPase [115]. They also have diverse vascular effects. While guanidine (which is not an inhibitor of NOS) and its methylated derivatives increase blood pressure, ethyl-, butyl-, and hexyl-guanidines cause a temporary hypotension before the pressor response [116–118]. Previous observations on vascular effects suggest that guanidino compounds with bulky substituents possess a smooth muscle relaxing action [116–118], whereas smaller guanidines may have pressor effects [116–118] that may be, at least partially, related to inhibition of NOS. *In vivo* administration of guanidines can result in tachyphylaxis: pressor responses to successive doses of the same guanidine diminish rapidly [116–118]. The depressor response, however, is unaffected [116–118]. The above factors should be born in mind when interpreting the cardiovascular effects of guanidines.

S-Substituted *isothioureas* (ITUs, Fig. 2c) have been described recently as very potent inhibitors of NOS by two independent groups [65, 109, 110]. Not only are some of these

* Southan GJ, Zingarelli B, O'Connor MP, Salzman AL and Szabo C, Manuscript submitted for publication.

compounds over 10–20 times more potent than L-NMA against iNOS, ecNOS, and nNOS, but they possess variable selectivity towards the various isoforms. *S*-Ethyl-ITU (Fig. 2c, R = $-CH_2CH_3$) and S-isopropyl-ITU appear to be the most potent members of the group of the straight chain ITUs. Extension of the side chain (R, Fig. 2c) decreases the potency (*n*-propyl > *t*-butyl > *n*-butyl, etc.) [110]. *S*-Ethyl- and *S*-isopropyl-ITU do not show any preference for a particular isoform in rodent models [110], but isopropyl-ITU shows some selectivity towards iNOS in human systems [109]. However, *S*-methyl-ITU is more potent on iNOS than on ecNOS in rodents [65]. Substitution of either or both of the amidine nitrogens greatly reduces the potency of a particular *S*-substituted ITU, as exemplified by *S*-methyl ITUs [110]. The exception to this is the cyclic compound, 2-aminothiazoline (where the sulfur is attached via a 2-carbon chain to one of the nitrogens) and its methylated derivatives, which are still as potent as L-NMA, but have no isoform selectivity. A further group of very potent ITUs are of the bis-ITU type where the sulfur atoms of two isothiourea units are linked by a carbon chain sometimes containing unsaturated (hetero)-cyclic rings. Some of these bis-ITUs show marked selectivity for human iNOS when compared with human ecNOS and bNOS preparations [109]. Unfortunately, poor cellular uptake and direct cytotoxic effects will probably limit the therapeutic usefulness of this "bis" class of compounds [109]. Isothioureas have been demonstrated to perturb the heme spectra of NOS, suggesting binding close to, or interaction with, the heme center [109].

So far, there are a limited number of published reports on *in vivo* experiments investigating the effects of ITUs in various models associated with NO overproduction. In a rat model of endotoxic shock, *S*-methyl-ITU caused a restoration of the blood pressure and pressor (vasoconstrictor) responsiveness. Its pressor effect (similar to that of aminoguanidine, but unlike L-NMA or L-NA) is larger in endotoxin-treated rats than in control animals [65]. Moreover, *S*-methyl-ITU reduces the endotoxin-induced increase in the plasma markers of hepatic injury [65], reduces endotoxin-induced pulmonary leak in rats [101], and improves 24-hr survival in endotoxin-treated mice [65]. Similarly, *S*-methyl-ITU has beneficial effects in a gram-positive model of sepsis in rats [119]. In a hyperdynamic model of endotoxin shock in the sheep, *S*-ethyl-ITU has been shown to cause a marked and sustained increase in blood pressure [120]. *S*-Methyl-ITU [61] and *S*-aminoethyl-ITU are also effective in preventing endotoxin-induced liver failure [111], although the effects of the latter drug are presumably due to its metabolite, mercaptoethylguanidine (as discussed above). Interestingly, there is an early report demonstrating a marked pressor effect of *S*-methyl-ITU in a patient with spinal anesthesia [121]. This pressor effect is likely to represent the consequence of the inhibitory effect of the drug on ecNOS, rather than iNOS.

S-Methyl-ITU or other derivatives may cause tachyphylaxis in as much as pressor responses to subsequent injections of the agent or of other ITUs are attenuated in anesthetized animals. Indeed, this phenomenon has been noted for related pressor agents (isoureas, amidines) [116, 117, 122]. Thus, it appears that ITUs, along with other derivatives of amidines, possess additional properties that affect the cardiovascular system.

Guanidines and isothioureas can be classified as derivatives of *amidines* (Fig. 2), of which formamidine (Fig. 2b, —SR=H) is the lowest member of the homologous series. Neither formamidine nor acetamidine (Fig. 2a, R=H) inhibits iNOS, but propionamidine (Fig. 2a, R = $-CH_3$) and longer chained amidines do [123]. Indeed, the cyclic amidine, 2-iminopiperidine, is a more potent inhibitor of iNOS than is L-NMA. Although some of these agents may have some preference for iNOS over ecNOS when compared with L-NMA, they are also powerful pressor agents in the rat, some disproportionately so when compared with their ability to inhibit NOS (e.g. butyramidine). Some amidines that are weak inhibitors of NOS are also potent pressor agents [116, 117], suggesting that these small amidines may exert their pressor actions through more than one mechanism. Long chain amidines (and long chain guanidines), on the other hand, have been observed to exhibit *de*pressor actions. Of potential relevance to their NOS inhibitory actions is the ability of some derivatives of amidines to blunt the pressor effects of subsequent injections of the same or different classes of amidine derivatives (including guanidines)—a cross-species tachyphylaxis [122].

From the group of imidazoles and indazoles, *7-nitroindazole* has received the most attention, as a selective inhibitor of bNOS. In contrast to other bNOS-selective agents, such as *S*-methyl- and *S*-ethyl-thiocitrulline or L-NA, the basis of selectivity appears to lie in the differential uptake of the inhibitor into endothelial cells versus neurons: 7-nitroindazole has minimal pressor effects *in vivo*, but is an extremely potent inhibitor of NOS activity in homogenates of endothelial cells [80, 81, 124]. The inhibition of NOS by 7-nitroindazole may involve multiple mechanisms: it binds to the heme group of NOS in an L-arginine and/or BH_4 reversible fashion, depending on the isoform of NOS studied [80], and so appears to affect both the pteridine and arginine binding sites of NOS [125]. The first striking biological action of 7-nitroindazole was its potent antinociceptive effect, which is related to inhibition of bNOS. Similar antinociceptive effects have been observed with L-NA but not L-NMA. There are characteristic changes in regional cerebral blood flow in animals treated with 7-nitroindazole that are distinct from the effect of inhibition of ecNOS activity [126]. Recently, 7-nitroindazole has been used successfully against bNOS to protect against neurotoxicity elicited either by middle cerebral artery occlusion [127] or by the administration of 1-methyl-4-phenylpyridinum (MTPT) [128] in murine models. 7-Nitroindazole also exhibits NO-independent vascular actions such as endothelium-independent relaxant effect of various arterial smooth muscles [129]. 7-Nitroindazole reduces neurogenic edema formation, presumably by blocking bNOS present in the peripheral nerves [130].

Of the structurally related imidazoles, imidazole itself and its phenyl-substituted derivatives have been shown to inhibit NOS activity [79, 80], although only 1-phenylimidazole shows any appreciable selectivity, with a K_i for macrophage iNOS fifty times lower than for bNOS or ecNOS. As with other imidazoles, 1-phenylimidazole appears to exert its inhibitory

effects by virtue of its ability to bind heme and prevent the binding and activation of oxygen. However, its binding characteristics vary with isoform, but it is competitive with BH_4 and/or L-arginine.

It is likely that many of these small nitrogen-containing molecules can interact with the heme of NOS. Imidazoles inhibit the activity of various heme-containing proteins by binding to the heme group. Aminoguanidine chelates copper and iron ions and inhibits the actions of many enzymes that contain them. However, compounds that chelate iron ions do not necessarily affect NOS activity (amidoximes, for example). In addition there must be steric/structural constraints in the close proximity to the heme. Indeed, the heme group may be regarded as a portion of the inhibitor binding site.

SUMMARY AND FUTURE DIRECTIONS

Clearly, the various pathophysiological conditions associated with alterations in the body's NO homeostasis make NOS an attractive target for drug development. However, the complexity of the actions of NO and the various isoforms of NOS makes it necessary to develop isoform-selective, or, ideally, even cell-selective inhibitors of NOS. Specific targeting of NOS activity may be achievable by: (1) targeting the specific co-factor requirement of the various isoforms (calmodulin, BH_4, etc.); (2) targeting the L-arginine uptake systems or the availability of L-arginine (in the case of iNOS); (3) exploiting the differential uptake and/or metabolic characteristics of cells that express NOS (e.g. 7-nitroindazole); or (4) developing agents that specifically suppress the catalytic activity of a selected isoform of NOS. In the past, the development of substrate antagonists as isoform selective inhibitors of NOS has focused on: (a) L-arginine-based inhibitors; (b) non-arginine-based, amino-acid derivative inhibitors; and more recently (c) small, non-amino acid-based inhibitors. All three approaches have been successful inasmuch as approach (a) yielded bNOS selective inhibitors such as L-NA and cyclopropyl-L-arginine, approach (b) yielded the iNOS selective L-N^6-(1-iminoethyl)lysine, and approach (c) resulted in the discovery of the iNOS selective guanidines (aminoguanidine and mercaptoalkylguanidines) and various isothiourea derivatives.

Approach (c) may have advantages in that non-arginine compounds may yield more information about the catalytic site of NOS: the stringent requirements for the binding of the amino acid group of an arginine analogue restricts the freedom of the (modified) guanidino group to arrange itself within the guanidine binding pocket of the substrate binding site. Small non-amino acid molecules, however, could arrange themselves within the guanidine/catalytic pocket independently of any such constraints. The point can be illustrated by the example of *S*-methisothiourea, a potent and somewhat selective inhibitor of iNOS: when a 2-aminopentanoic acid side chain is attached to one of the nitrogens (to give *S*-methyl-L-thiocitrulline), the selectivity is altered in favor of bNOS. When the side chain is attached to the sulfur in place of the methyl group to make an arginine analogue (that is, the ϵ-nitrogen of arginine is replaced by a sulfur), the potency is substantially reduced.

Future Trends

From recent reports it seems that many groups believe that the achievement of pharmacological inhibitors of NOS with isoform selectivity is a realistic goal. Isoform selective NOS inhibitors may have therapeutic and commercial value in the therapy of various inflammatory and neurodegenerative disorders. It is generally assumed that the amino acid group of L-arginine analogues binds to a pocket within the substrate binding site that exhibits fairly stringent steric requirements. However, there is no evidence to suggest that this binding *per se* imparts any isoform-selectivity to any amino acid inhibitor of NOS. As it seems unnecessary to incorporate a group that is equally well recognized by all isoforms of NOS into the structure of a potential selective inhibitor, it is the opinion of the authors that the study of non-amino acid amidine derivatives will be at least as productive as the continued study of arginine analogues in the search for selective inhibitors of iNOS. The difference between the isoform preferences of aminoguanidine and its arginine derivative. N^G-amino-L-arginine, is one example to illustrate this point. Aminoguanidine also demonstrates the potential for more general actions of non-arginine-based compounds that are unrelated to their effects on NOS. However, as mentioned above, L-arginine-based inhibitors of NOS also have numerous side-effects. Such effects have to be considered when interpreting experimental data obtained with NOS inhibitors of the various structural classes.

The discovery of the inhibitory properties of certain isothioureas and nitroindazoles has raised interest in non-amino acid compounds. Most inhibitors of NOS thus far reported contain the amidine function ($-C(-NH_2)=NH$). This group encompasses arginines, guanidines, isothioureas, amidines, and even imidazoles. As the number of inhibitor types broadens, more will be learned about the structural features consistent with selective inhibition of NOS isoforms. Moreover, structural features deemed significant in one class of compounds may be subsequently tried in others. In view of the respective effects of amidines and the bis-isothioureas on iNOS, for example, it is likely that analogous bis-amidines will be synthesized and found to be inhibitors of NOS. Similarly, the significance of sulfur in inhibitors (as in isothioureas and mercaptoalkylguanidines) will no doubt be investigated, spawning compounds with both amidine and sulfur (or possibly selenium) containing functionalities.

Another advantage in using small molecules is that the synthetic chemistry involved is usually less tedious and may encourage a wider range of groups to develop their thoughts on NOS inhibitors. Hopefully, this review may do likewise.

NOTE ADDED IN PROOF

We note with interest that a recent paper presented at the 4th "Biology of Nitric Oxide" meeting describes certain cyclic amidines as selective inhibitors of iNOS, and certain sulfur-containing amidines as selective inhibitors of bNOS [131].

References

1. Nathan C, Nitric oxide as a secretory product of mammalian cells. *FASEB J* **6:** 3051–3064, 1992.
2. Marletta MA, Mammalian synthesis of nitrite, nitrate, nitric oxide and N-nitrosating agents. *Chem Res Toxicol* **1:** 249–257, 1988.
3. Marletta MA, Nitric oxide synthase structure and mechanism. *J Biol Chem* **268:** 12231–12234, 1993.
4. Vane JR, The Croonian lecture, 1993: The endothelium: Maestro of the blood circulation. *Proc R Soc Lond B* **343:** 225–246, 1994.
5. Dinerman JL, Lowenstein CJ and Snyder SH, Molecular mechanisms of nitric oxide production. Potential relevance to cardiovascular disease. *Circ Res* **73:** 217–222, 1993.
6. Snyder S and Bredt DS, Biological roles of nitric oxide. *Sci Am* **166:** 28–35, 1992.
7. Dawson VL, Dawson TM, London ED, Bredt DS and Snyder SH, Nitric oxide mediates glutamate neurotoxicity in primary cortical cultures. *Proc Natl Acad Sci USA* **88:** 6368–6371, 1991.
8. Garthwaite J, Glutamate, nitric oxide and cell–cell signalling in the nervous system. *Trends Neurosci* **14:** 60–67, 1991.
9. Xie Q and Nathan C, The high-output nitric oxide pathway: Role and regulation. *J Leukoc Biol* **56:** 576–582, 1994.
10. Kilbourn RG and Griffith OW, Overproduction of nitric oxide in cytokine-mediated and septic shock. *J Natl Cancer Inst* **84:** 827–831, 1992.
11. Palmer RMJ, The discovery of nitric oxide in the vessel wall. A unifying concept in the pathogenesis of sepsis. *Arch Surg* **128:** 396–401, 1993.
12. Szabó C and Thiemermann C, Invited opinion: Role of nitric oxide in hemorrhagic, traumatic and anaphylactic shock and in thermal injury. *Shock* **2:** 145–155, 1994.
13. Szabó C, Alterations in nitric oxide production in various forms of circulatory shock. *New Horizons* **3:** 2–32, 1995.
14. McCartney-Francis N, Allen JN, Mizel DE, Albina J, Xie QW, Nathan CF and Wahl SM, Suppression of arthritis by an inhibitor of nitric oxide synthase. *J Exp Med* **178:** 749–753, 1993.
15. Miller MJS, Sadowska-Krowicka H, Chotinaruemol S, Kakkis JL and Clark DA, Amelioration of chronic iletis by nitric oxide synthase inhibition. *J Pharmacol Exp Ther* **264:** 11–16, 1993.
16. Chen P, Tsai AL and Wu K, Cysteine 184 of endothelial nitric oxide synthase is involved in heme coordination and catalytic activity. *J Biol Chem* **269:** 25062–25066, 1994.
17. McMillan K and Masters BSS, Prokaryotic expression of the heme- and flavin-binding domains of rat neuronal nitric oxide synthase as distinct polypeptides: Identification of the heme-binding proximal thiolate ligand as cysteine-415. *Biochemistry* **34:** 3686–3693, 1995.
18. Stuehr DJ, Fasehun OA, Kwon NS, Gross SS, Gonzalez JA, Levi R and Nathan CF, Inhibition of macrophage and endothelial cell nitric oxide synthase by diphenyleneiodonium and its analogs. *FASEB J* **5:** 98–103, 1991.
19. Nishimura JS, Martasek P, McMillan K, Salerno J, Liu Q, Gross SS, Masters BSS, Modular structure of neuronal nitric oxide synthase: Localization of the arginine binding site and modulation by pterin. *Biochem Biophys Res Commun* **210:** 288–294, 1995.
20. Feldman PL, Griffith OW, Honh H and Stuehr D, Irreversible inactivation of macrophage and brain nitric oxide synthase by L-N^G-methylarginine requires NADPH-dependent hydroxylation. *J Med Chem* **36:** 491–496, 1993.
21. Korth H-G, Sustmann R, Thater C, Butler AR and Ingold KU, On the mechanism of the nitric oxide synthase-catalyzed conversion of N^{ω}-hydroxy-L-arginine to citrulline and nitric oxide. *J Biol Chem* **269:** 17776–17779, 1995.
22. Fukuto JM and Chaudhuri G, Inhibition of constitutive and inducible nitric oxide synthase. Potential selective inhibition. *Annu Rev Pharmacol Toxicol* **35:** 165–194, 1995.
23. Komori Y, Wallace G and Fukuto J, Inhibition of purified nitric oxide synthase from rat cerebellum and macrophage by L-arginine analogs. *Arch Biochem Biophys* **315:** 213–218, 1994.
24. Yokoi I, Kabuto H, Habu H, Inada K, Toma J and Mori A, Structure–activity relationships of arginine analogues on nitric oxide synthase activity in the rat brain. *Neuropharmacology* **33:** 1261–1265, 1994.
25. Archer SL and Hampl V, N^G-Monomethyl-L-arginine causes nitric oxide synthesis in isolated arterial rings: Trouble in paradise. *Biochem Biophys Res Commun* **188:** 590–596, 1992.
26. Heinzel B, John M, Klatt P, Böhme E and Mayer B, Ca^{2+}/calmodulin-dependent formation of hydrogen peroxide by brain nitric oxide synthase. *Biochem J* **281:** 627–630, 1992.
27. Nussler AK, Billiar TR, Liu ZZ and Morris SM, Coinduction of nitric oxide synthase and arginosuccinate synthetase in a murine macrophage cell line. *J Biol Chem* **269:** 1257–1261, 1994.
28. Pacitti AJ, Copeland EM and Souba WW, Stimulation of hepatocyte system Y^+ mediated L-arginine transport by an inflammatory agent. *Surgery* **112:** 403–411, 1992.
29. Schott CA, Vetrovsky P and Stoclet JC, Cationic amino acids inhibit the effects of L-arginine in rat aorta exposed to lipopolysaccharide. *Eur J Pharmacol* **236:** 155–157, 1993.
30. Bogle RG, Moncada S, Pearson JD and Mann GE, Identification of inhibitors of nitric oxide synthase that do not interact with the endothelial cell L-arginine transporter. *Br J Pharmacol* **105:** 768–770, 1992.
31. Griffith OW, Park KH, Aisaka K, Levi R and Gross SS, The role of plasma arginine in nitric oxide synthesis: Studies with arginase-treated guinea pigs and rats. In: *The Biology of Nitric Oxide. I. Physiological and Clinical Aspects* (Eds. Moncada S, Marletta MA, Hibbs JB and Higgs EA), pp. 6–9. Portland Press, London, 1992.
32. Bianchi M, Bloom O, Meistrell M, Ulrich A, Cerami A and Tracey KJ, Development of a selective inhibitor of cytokine-inducible L-arginine transport (CNI-1493) that blocks macrophage production of NO and TNFα and protects against LPS lethality. *Shock* **3**(Suppl 2): 9, 1995.
33. Schini VB and Vanhoutte PM, Inhibitors of calmodulin impair the constitutive but not the inducible nitric oxide synthase activity in the rat aorta. *J Pharmacol Exp Ther* **261:** 553–559, 1992.
34. Jorens PG, Overveld FJ, Bult H, Vermeire PA and Herman AG, Pterins inhibit nitric oxide synthase activity in rat alveolar macrophages. *Br J Pharmacol* **107:** 1088–1091, 1992.
35. Gross SS and Levi R, Tetrahydrobiopterin synthesis. An absolute requirement for cytokine-induced nitric oxide generation by vascular smooth muscle. *J Biol Chem* **267:** 25722–25729, 1992.
36. Schoedon G, Blau N, Schneemann M, Flury G and Schaffner A, Nitric oxide production depends on preceding tetrahydrobiopterin synthesis by endothelial cells: Selective suppression of induced nitric oxide production by sepiapterin reductase inhibitors. *Biochem Biophys Res Commun* **199:** 504–510, 1994.
37. Klemm P, Ostrowski J, Morath T, Gruber C, Martorana PA and Henning R, *N*-Acetylserotonin prevents the hypotension induced by bacterial lipopolysaccharides in the rat. *Eur J Pharmacol* **250:** R9–R10, 1993.
38. Klemm P, Hecker M, Stockhausen H, Wu CC and Thiemermann C, Inhibition by *N*-acetyl-5-hydroxytryptamine of nitric oxide synthase expression in cultured cells and in the anaesthetized rat. *Br J Pharmacol* **115:** 1175–1181, 1995.
39. Levine RA, Miller LP and Lovenberg W, Tetrahydrobiopterin in striatum: Localization in dopamine nerve terminals and role in catecholamine synthesis. *Science* **214:** 919–921, 1981.
40. Zimmerman TP, Nichol CA and Viveros OH, Regulation of

guanosine triphosphate cyclohydrolase and tetrahydrobiopterin levels and the role of the cofactor in tyrosine hydroxylation in primary cultures of adrenomedullary chromaffin cells. *J Neurochem* **46:** 1190–1199, 1986.
41. Kapatos G, Hirayama K and Hasegawa H, Tetrahydrobiopterin turnover in cultured rat sympathetic neurons: Developmental profile, pharmacologic sensitivity, and relationship to norepinephrine synthesis. *J Neurochem* **59:** 2048–2055, 1992.
42. Dawson TM, Steiner JP, Dawson VL, Dinerman JL, Uhl G and Snyder S, Immunosuppressant FK506 enhances phosphorylation of nitric oxide synthase and protects against glutamate neurotoxicity. *Proc Natl Acad Sci USA* **90:** 9808–9812, 1993.
43. Moncada S, Palmer RMJ and Higgs EA, Nitric oxide: Physiology, pathophysiology, and pharmacology. *Pharmacol Rev* **43:** 109–141, 1991.
44. Wang Y-X, Poon CI and Pang CCY, *In vitro* and *ex vivo* inhibitory effects of L- and D-enantiomers of N^G-nitro-arginine on endothelium-dependent relaxation of rat aorta. *J Pharmacol Exp Ther* **265:** 112–119, 1993.
45. Southan GJ, Gross SS and Vane JR, Esters and amides of N^G-nitro-L-arginine act as prodrugs in their inhibition of inducible nitric oxide syntase. In: *The Biology of Nitric Oxide. 4: Enzymology, Biochemistry and Immunology* (Eds. Moncada S, Feelisch M, Busse R and Higgs EA), pp. 4–7. Portland Press, London, 1995.
46. Lambert LE, French JF, Whitten JP, Baron BM and McDonald IA, Characterization of cell selectivity of two novel inhibitors of nitric oxide synthesis. *Eur J Pharmacol* **216:** 131–134, 1992.
47. Gross SS, Jaffe EA, Levi R and Kilbourn RG, Cytokine-activated endothelial cells express an isotype of nitric oxide synthase which is tetrahydrobiopterin-dependent, calmodulin-independent and inhibited by arginine analogs with a rank-order of potency characteristic of activated macrophages. *Biochem Biophys Res Commun* **187:** 823–829, 1991.
48. Lambert LE, Whitten JP, Baron BM, Cheng HC, Doherty NS and McDonald IA, Nitric oxide synthesis in the CNS, endothelium and macrophages differs in its sensitivity to inhibition by arginine analogues. *Life Sci* **48:** 69–75, 1991.
49. Gross SS, Stuehr DJ, Aisaka K, Jaffe EA, Levi R and Griffith OW, Macrophage and endothelial cell nitric oxide synthesis: Cell-type selective inhibition by N^G-aminoarginine, N^G-nitroarginine and N^G-methylarginine. *Biochem Biophys Res Commun* **170:** 96–103, 1990.
50. Furfine ES, Harmon MF, Paith JE and Garvey EP, Selective inhibition of constitutive nitric oxide synthase by L-N^G-nitroarginine. *Biochemistry* **32:** 8512–8517, 1993.
51. Abu-Soud H, Feldman P, Clark P and Stuehr D, Electron transfer in the nitric-oxide synthases. *J Biol Chem* **269:** 32318–32326, 1994.
52. McCall TB, Feelisch M, Palmer RMJ and Moncada S, Identification of *N*-iminoethyl-L-ornithine as an irreversible inhibitor of nitric oxide synthase in phagocytic cells. *Br J Pharmacol* **102:** 234–238, 1991.
53. Olken NM, Osawa Y and Marletta MA, Characterization of the inactivation of nitric oxide synthase by N^G-methyl-L-arginine: Evidence for heme loss. *Biochemistry* **33:** 14784–14791, 1994.
54. Klatt P, Schmidt K, Brunner F and Mayer B, Inhibitors of brain nitric oxide synthase: Binding kinetics, metabolism and enzyme inactivation. *J Biol Chem* **269:** 1674–1680, 1994.
55. Vallance P, Leone A, Calver A, Collier J and Moncada S, Accumulation of endogenous inhibitor of nitric oxide synthesis in chronic renal failure. *Lancet* **393:** 575–577, 1992.
56. MacAllister RJ, Whitley GS and Vallance P, Effects of guanidino and uremic compounds on nitric oxide pathways. *Kidney Int* **45:** 737–742, 1994.
57. Yu XJ, Li YJ and Xong Y, Increase of an endogenous inhibitor of nitric oxide synthesis in serum of high cholesterol fed rabbits. *Life Sci* **54:** 753–758, 1994.
58. Stefanovic-Racic M, Stadler J and Evans CH, Nitric oxide and arthritis. *Arthritis Rheu* **36:** 1036–1044, 1993.
59. Nava E, Palmer RMJ and Moncada S, Inhibition of nitric oxide synthesis in septic shock: How much is beneficial? *Lancet* **338:** 1555–1557, 1991.
60. Nava E, Palmer RMJ and Moncada S, The role of nitric oxide in endotoxic shock: Effects of N^G-monomethyl-L-arginine. *J Cardiovasc Pharmacol* **20**(Suppl 12): S132–S134, 1992.
61. Lukic ML, Stosic-Grujicic S, Ostojic N, Chan WL and Liew FY, Inhibition of nitric oxide generation affects the induction of diabetes by streptozocin in mice. *Biochem Biophys Res Commun* **178:** 913–920, 1991.
62. Kolb H, Kiesel U, Kroncke KD and Bachofen V, Suppression of low dose streptozocin induced diabetes in mice by administration of a nitric oxide synthase inhibitor. *Life Sci* **49:** PL213–PL217, 1991.
63. Meyer J, Traber LD, Nelson S, Lentz CW, Nakazawa H, Herndon DN, Noda H and Traber DL, Reversal of hyperdynamic response to continuous endotoxin administration by inhibition of NO synthesis. *J Appl Physiol* **73:** 324–328, 1992.
64. Laszlo F, Whittle BJR and Moncada S, Time-dependent enhancement or inhibition of endotoxin-induced vascular injury in rat intestine by nitric oxide synthase inhibitors. *Br J Pharmacol* **111:** 1309–1315, 1994.
65. Szabó C, Southan GJ and Thiemermann C, Beneficial effects and improved survival in rodent models of septic shock with *S*-methyl-isothiourea sulfate, a novel, potent and selective inhibitor of inducible nitric oxide synthase. *Proc Natl Acad Sci USA* **91:** 12472–12476, 1994.
66. MacMicking JD, Nathan C, Hom G, Chartrain N, Fletcher DS, Trumbauer M, Stevens K, Xie Q-W, Sokol K, Hutchinson N, Chen H and Mudgett JS, Altered responses to bacterial infection and endotoxic shock in mice lacking inducible nitric oxide synthase. *Cell* **81:** 641–650, 1995.
67. Billiar TR, Curran RD, Harbrecht BG, Stuehr DJ, Demetris AJ and Simmons RL, Modulation of nitrogen oxide synthesis *in vivo*: N^G-monomethyl-L-arginine inhibits endotoxin-induced nitrite/nitrate biosynthesis while promoting hepatic damage. *J Leukoc Biol* **48:** 565–569, 1990.
68. Robertson CA, Green BG, Niedzwiecki L, Harrison L and Grant SK, Effect of nitric oxide synthase inhibitors on rat liver arginase. *Biochem Biophys Res Commun* **197:** 523–528, 1993.
69. Frew JD, Paisley K and Martin W, Selective inhibition of basal but not agonist-stimulated activity of nitric oxide in rat aorta by N^G-monomethyl-L-arginine. *Br J Pharmacol* **110:** 1003–1008, 1993.
70. Buxton ILO, Cheek DJ, Eckman D, Westfall DP, Sanders KM and Keef KD, N^G-Nitro-L-arginine methyl ester and other alkyl esters of arginine are muscarinic receptor antagonists. *Circ Res* **72:** 387–395, 1993.
71. Rotzinger S, Aragon CMG, Rogan F, Amir S and Amit Z, The nitric oxide synthase inhibitor N^ω-nitro-L-arginine methylester attenuates brain catalase activity *in vitro*. *Life Sci* **56:** 1321–1324, 1995.
72. Peterson DA, Peterson DC, Archer S and Weir EK, The non-specificity of specific nitric oxide synthase inhibitors. *Biochem Biophys Res Commun* **187:** 797–801, 1992.
73. Cobb JP, Natanson C, Hoffman WD, Lodato RF, Banks S, Koev CA, Solomon MA, Elin RJ, Hosseini JM and Danner RL, N^ω-Amino-L-arginine, an inhibitor of nitric oxide synthase, raises vascular resistance but increases mortality rates in awake canines challenged with endotoxin. *J Exp Med* **176:** 1175–1182, 1992.
74. Furfine ES, Harmon MF, Paith JE, Knowles RG, Salter M, Kiff RJ, Duffy C, Hazelwood R, Oplinger JA and Garvey EP, Potent and selective inhibition of human nitric oxide synthases. Selective inhibition of neuronal nitric oxide synthase by *S*-meth-

yl-L-thiocitrulline and S-ethyl-L-thiocitrulline. *J Biol Chem* **269:** 26677–26683, 1994.
75. Narayanan K and Griffith O, Synthesis of L-thiocitrulline, L-homothiocitrulline, and S-methyl-L-thiocitrulline: A new class of potent nitric oxide synthase inhibitors. *J Med Chem* **37:** 885–887, 1994.
76. Moore WM, Webber RK, Jerome GM, Tjoeng FS, Misko TP and Currie MG, L-N^6-(1-Iminoethyl)lysine: A selective inhibitor of inducible nitric oxide synthase. *J Med Chem* **37:** 3886–3888, 1994.
77. Frey C, Narayanan K, McMillan K, Spack L, Gross S, Masters B and Griffith O, L-Thiocitrulline. A stereospecific, heme-binding inhibitor of nitric-oxide synthases. *J Biol Chem* **269:** 26083–26091, 1994.
78. Connor JR, Manning PT, Settle SL, Moore WM, Jerome GM, Webber RK, Tjoeng FS and Currie MG, Suppression of adjuvant-induced arthritis by selective inhibition of inducible nitric oxide synthase. *Eur J Pharmacol* **273:** 15–24, 1995.
79. Wolff DJ, Datto GA, Samatowitz RA and Tempsick RA, Calmodulin-dependent nitric oxide synthase. Mechanism of inhibition by imidazole and phenylimidazoles. *J Biol Chem* **268:** 9425–9429, 1993.
80. Wolff DJ, Lubeskie A and Umansky C, The inhibition of the constitutive bovine endothelial nitric oxide synthase by imidazole and indazole agents. *Arch Biochem Biophys* **314:** 360–366, 1994.
81. Moore PK, Wallace P, Gaffen Z, Hart SL and Babbedge RC, Characterization of the novel nitric oxide synthase inhibitor 7-nitro indazole and related indazoles: Antinociceptive and cardiovascular effects. *Br J Pharmacol* **110:** 219–224, 1993.
82. Hasan K, Heesen BJ, Corbett JA, McDaniel ML, Chang K, Allison W, Wolfenbuttel BHR, Williamson JR and Tilton RG, Inhibition of nitric oxide formation by guanidines. *Eur J Pharmacol* **249:** 101–106, 1993.
83. Sup SJ, Green BG and Grant SK, 2-Iminobiotin is an inhibitor of nitric oxide synthases. *Biochem Biophys Res Commun* **204:** 962–968, 1994.
84. Hatchett RJ, Gryglewski RJ, Mlochowski J, Zembowicz A and Radziszewski W, Carboxyebselen a potent and selective inhibitor of endothelial nitric oxide synthase. *J Physiol Pharmacol* **45:** 55–67, 1994.
85. Yokoi I, Kabuto H, Habu H and Mori A, α-Guanidinoglutaric acid, an endogenous convulsant, as a novel nitric oxide synthase inhibitor. *J Neurochem* **63:** 1565–1567, 1994.
86. Chiesi M and Schwaller R, Inhibition of constitutive endothelial NO synthase activity by tannin and quercetin. *Biochem Pharmacol* **49:** 495–501, 1995.
87. Buchmüller-Rouiller Y, Schneider P, Betz-Corradin S, Smith J and Mauél, 3-Amino-1,2,4-triazole inhibits macrophage NO synthase. *Biochem Biophys Res Commun* **183:** 150–155, 1992.
88. Osawa Y and Davila J, Phencyclidine, a psychotomimetic agent and drug of abuse, is a suicide inhibitor of brain nitric oxide synthase. *Biochem Biophys Res Commun* **194:** 1435–1439, 1993.
89. Mayer B, Brunner F and Schmidt B, Inhibition of nitric oxide synthesis by methylene blue. *Biochem Pharmacol* **45:** 367–374, 1993.
90. Corbett A, Tilton RG and Chang K, Aminoguanidine, a novel inhibitor of nitric oxide formation, prevents diabetic vascular dysfunction. *Diabetes* **41:** 552–556, 1992.
91. Wu C-C, Chen S-J, Szabó C, Thiemermann C and Vane JR, Aminoguanidine attenuates the delayed circulatory failure and improves survival in rodent models of endotoxic shock. *Br J Pharmacol* **114:** 1666–1672, 1995.
92. Joly GA, Ayres M, Chelly F and Kilbourn RG, Effects of N^G-methyl-L-arginine, N^G-nitro-L-arginine and aminoguanidine on constitutive and inducible nitric oxide synthase in rat aorta. *Biochem Biophys Res Commun* **199:** 147–154, 1994.
93. Laszlo F, Evans SM and Whittle BJR, Aminoguanidine inhibits both constitutive and inducible nitric oxide synthase isoforms in rat intestinal microvasculature *in vivo*. *Eur J Pharmacol* **272:** 169–175, 1994.
94. Wu G, Nitric oxide synthesis and the effect of aminoguanidine and N^G-monomethyl-L-arginine on the onset of diabetes in the spontaneously diabetic BB rat. *Diabetes* **44:** 360–364, 1995.
95. Cross AH, Misko TP, Lin RF, Hickey WF, Trotter JL and Tilton RG, Aminoguanidine, an inhibitor of inducible nitric oxide synthase, ameliorates experimental autoimmune encephalomyelitis in SJL mice. *J Clin Invest* **93:** 2684–2690, 1994.
96. Misko TP, Moore WM, Kasten TP, Nickols DA, Corbett JA, Tilton RG, McDaniel ML, Williamson JR and Currie MG, Selective inhibition of the inducible NO synthase by aminoguanidine. *Eur J Pharmacol* **233:** 119–125, 1993.
97. Tilton RG, Chang K, Hasan KS, Smith SR, Petrash JM, Misko TP, Moore WM, Currie MG, Corbett JA, McDaniel ML and Williamson JR, Prevention of diabetic vascular dysfunction by guanidines. Inhibition of nitric oxide synthase versus advanced glycation end-product formation. *Diabetes* **42:** 221–232, 1993.
98. Holstad M and Sandler S, Aminoguanidine, an inhibitor of nitric oxide formation, fails to protect against insulitis and hyperglycemia induced by multiple low dose streptozotocin injections in mice. *Autoimmunity* **15:** 311–314, 1993.
99. Griffiths MJ, Messent M, MacAllister RJ and Evans TW, Aminoguanidine selectively inhibits inducible nitric oxide synthase. *Br J Pharmacol* **110:** 963–968, 1993.
100. Lomis TJ, Siffring CW, Chalasani S, Ziegler DW, Lentz KE, Stauffer KE, McMillan A, Agarwal N, Lowenstein CJ and Rhoads JE Jr, Nitric oxide synthase inhibitors *N*-monomethylarginine and aminoguanidine prevent the progressive and severe hypotension associated with a rat model of pancreatitis. *Am Surg* **61:** 7–10, 1995.
101. Arkowitz M, Szabó C, Wispe JR and Garcia VF, Selective inhibition of the inducible isoform of nitric oxide synthase inhibits endotoxin-induced pulmonary leak. *J Pediatr Surg*, in press.
102. Bieganski T, Kusche J, Lorenz W, Hesterberg R, Stahlknecht CD and Feussner KD, Distribution and properties of human intestinal diamine oxidase and its relevance for the histamine catabolism. *Biochem Biophys Acta* **756:** 196–203, 1983.
103. Seiler N, Bolkenius FN and Knodgen B, The influence of catabolic reactions on polyamine excretion. *Biochem J* **225:** 219–226, 1985.
104. Ou P and Wolff SP, Aminoguanidine: A drug proposed for prophylaxis in diabetes inhibits catalase and generates hydrogen peroxide *in vitro*. *Biochem Pharmacol* **46:** 1139–1144, 1993.
105. Picard S, Parthasarathy S, Fruebis J and Witztum JL, Aminoguanidine inhibits oxidative modification of low density lipoprotein protein and the subsequent increase in uptake by macrophage scavenger receptors. *Proc Natl Acad Sci USA* **89:** 6876–6880, 1992.
106. Eaton JD, Stevens EJ and Tomlinson DR, Effects of aminoguanidine on prostacyclin release and interactions with L-NAME. *Br J Pharmacol* **106:** P8, 1992.
107. Wolff DJ and Lubeskie A, Aminoguanidine is an isoform-selective, mechanism-based inactivator of nitric oxide synthase. *Arch Biochem Biophys* **316:** 290–301, 1995.
108. Southan GJ, Salzman AL and Szabó C, Merkaptoalkyle guanidines: A novel class of inhibitors of the inducible isoform of nitric oxide synthase. *FASEB J* **8:** A28, 1995.
109. Garvey EP, Oplinger JA, Tanoury GJ, Sherman PA, Fowler M, Marshall S, Harmon MF, Patih JE and Furfine ES, Potent and selective inhibition of human nitric oxide synthases. *J Biol Chem* **269:** 26669–26676, 1994.
110. Southan GJ, Szabó C and Thiemermann C, Isothioureas: Potent inhibitors of nitric oxide synthases with variable isoform selectivity. *Br J Pharmacol* **114:** 510–516, 1995.

111. Thiemermann C, Rutten H, Wu CC and Vane JR, Nitric oxide and organ failure. *Shock* **3**(Suppl 1): S35, 1995.
112. Kozak A and Arient M, Metabolism and radioprotective effect of AET and MEG in organism. *Strahlentherapie* **146:** 367–373, 1973.
113. Shapira R, Doherty DG and Burnett WT, Chemical protection against ionizing radiation. III. Mercaptoalkylguanidines and related isothiouronium compounds with protective activity. *Radiat Res* **7:** 22–34, 1957.
114. Liebmann J, DeLuca AM, Coffin D, Keefer LK, Venzon D, Wink DA and Mitchell JB, *In vivo* radiation protection by nitric oxide modulation. *Cancer Res* **54:** 3365–3368, 1994.
115. David P, Mayan H, Tal DM and Karlish SJD, Guanidium derivatives act as high affinity antagonists of Na^+ ions in occlusion sites of Na^+,K^+-ATPase. *J Biol Chem* **267:** 1141–1149, 1992.
116. Fastier FN and Smirk F, Circulatory properties of iso-thioureas, guanidines, iso-ureas and amidines. *J Pharmacol* **89:** 256–270, 1947.
117. Fastier FN, Structure–activity relationships of amidine derivatives. *Pharmacol Rev* **14:** 37–90, 1962.
118. Ozawa H and Sugawara K, Structure–activity relationships of alkylguanidines on smooth muscle organs. *Chem Pharm Bull (Tokyo)* **16:** 2376–2382, 1968.
119. Kengatharan M, Szabó C, DeKimpe S, Southan GJ and Thiemermann C, S-methyl-isothiourea, a potent inhibitor of the inducible isoform of nitric oxide synthase, has beneficial hemodynamic effects in gram positive and gram negative forms of circulatory shock. *FASEB J* **8:** A28, 1995.
120. Booke M, Schenarts PJ, Shin C, Biondo N, McGuire R, Traber LD and Traber DL, A new non-amino acid inhibitor of nitric oxide synthase (3936W920) in hyperdynamic ovine sepsis. *Shock* **3**(Suppl 1): S72, 1995.
121. Smirk FH and McGeorge M, S-Methylisothiourea sulphate for maintaining blood-pressure in spinal anaesthesia. *Lancet* **2:** 301–302, 1942.
122. Fastier FN and Smirk FH, The circulatory effects of some iso-thiourea derivatives, with special reference to the sensitization of animals. *J Physiol (Lond)* **101:** 379–388, 1943.
123. Southan GJ, Szabó C, O'Connor MP, Salzman AL and Thiemermann C, Amidines are potent inhibitors of nitric oxide synthases: Preferential inhibition of the inducible isoform. *Eur J Pharmacol*, in press.
124. Babbedge RC, Bland-Ward PA, Hart SL and Moore PK, Inhibition of rat cerebellar nitric oxide synthase by 7-nitroindazole and related substituted indazoles. *Br J Pharmacol* **110:** 225–228, 1993.
125. Mayer B, Klatt P, Werner ER and Schmidt K, Molecular mechanisms of inhibition of porcine brain nitric oxide synthase by the antinociceptive drug 7-nitro-indazole. *Neuropharmacology* **33:** 1253–1259, 1994.
126. Kovach AGB, Lohinai Z, Balla I, Marczis J, Dombovary Z, Reivich M, Dawson TM and Synder SH, Effect of 7-nitro indazole on rCBF, cerebral NOS catalytic activity and NO content measured by microelectrode. *Endothelium* **1:** S54, 1993.
127. Yoshida T, Limmroth V, Irikura K and Moskowitz MA, The NOS inhibitor, 7-nitroindazole, decreases focal infarct volume but not the response to topical acetylcholine in pial vessels. *J Cereb Blood Flow Metab* **14:** 924–929, 1994.
128. Schulz JB, Matthews RT, Muqit MM, Browne SE and Beal MF, Inhibition of neuronal nitric oxide synthase by 7-nitroindazole protects against MPTP-induced neurotoxicity in mice. *J Neurochem* **64:** 936–939, 1995.
129. Medhurst AD, Greenlees C, Parsons AA and Smith SJ, Nitric oxide synthase inhibitors 7- and 6-nitroindazole relax smooth muscle *in vitro*. *Eur J Pharmacol* **256:** R5–R6, 1994.
130. Kajekar R, Moore PK and Brain SD, Essential role for nitric oxide in neurogenic inflammation in rat cutaneous microcirculation. Evidence for an endothelium-independent mechanism. *Circ Res* **76:** 441–447, 1995.
131. Hansen OW, Peterson KB, Trivedi M, Moore WM, Currie MG, Manning PT, Connor JR and Pitzele BS, Homopiperidine amidines as selective inhibitors of inducible nitric oxide synthase. *Endothelium* **3:** (Suppl.) S80, 1995.