Biochemistry (Moscow), Vol. 70, No. 1, 2005, pp. 8-23. Translated from Biokhimiya, Vol. 70, No. 1, 2005, pp. 14-32.

REVIEW

Structure and Activity of NO Synthase Inhibitors Specific to the L-Arginine Binding Site

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Received December 26, 2003
Revision received April 12, 2004

Abstract—Synthesis of compounds containing a fragment similar to the guanidine group of L-arginine, which is a substrate of nitric oxide synthase (NOS), is the main direction in creating NOS inhibitors. The inhibitory effect of such compounds is caused not only by their competition with the substrate for the L-arginine-binding site and/or oxidizing center of the enzyme (heme) but also by interaction with peptide motifs of the enzyme that influence its dimerization, affinity for cofactors, and interaction with associated proteins. Structures, activities, and relative *in vitro* and *in vivo* specificities of various NOS inhibitors (amino acid and non-amino acid) with linear or cyclic structure and containing guanidine, amidine, or isothiuronium group are considered. These properties are mainly analyzed by comparison with effects of the inhibitors on the inducible NOS.

Key words: nitric oxide synthase (NOS), NOS activity regulation, reactive oxygen species, substrate-like inhibitors, chemical structure, activity and selectivity of NOS inhibitors

The search for inhibitors more active and selective toward a particular nitric oxide synthase (NOS) isoform is still a current problem [1, 2]. Such inhibitors are promising for treatment of inflammatory diseases (arthritis, diabetes mellitus, myocardial ischemia [3, 4]), hypotension-associated shocks [5, 6], and also as radiomodifiers [7] and chemoprotective agents [8]. This paper presents a review of biochemical targets promising as substrate-like inhibitors and of new classes of chemical structures among which the search for effective compounds is performed. Properties of the inhibitors will be considered mainly in comparison to the inducible NOS (iNOS), which plays not only a key role in the nonspecific immunity [9] but can also suppress protective mechanisms, in particular, those mediated by endothelial NOS (eNOS) in sepsis [10, 11] and ischemia–reperfusion [12, 13].

STRUCTURE AND FUNCTIONS OF NOS

NOS is a family of enzymes which are regulated by numerous biochemical systems because of the multiform nature of NO$^{\bullet}$ [14, 15].

Substrates and cofactors. NO$^{\bullet}$ is synthesized under the influence of a homodimeric enzyme with involvement of three substrates: L-arginine, oxygen, and NADPH as a source of electrons. The NOS activity is provided by a number of cofactors and prosthetic groups. Some of them are components of each subunit: FAD, FMN, heme with pentacoordinated Fe^{2+}, the Ca^{2+}-binding protein calmodulin (CaM), Zn^{2+}, and according to many data, (6R)-5,6,7,8-tetrahydro-L-biopterin (BH_4), which can be unique in the dimer. Flavins of the reductase domain carry

Abbreviations: NO$^{\bullet}$) nitric oxide; $O_2^{\bullet-}$) superoxide anion radical; ROS) reactive oxygen species; LPS) lipopolysaccharide; NOS) nitric oxide synthase; nNOS) neuronal NOS; iNOS) inducible NOS; eNOS) endothelial NOS; m-, r-, b-, and h-NOS) mouse, rat, bovine, and human NOS; CaM) calmodulin; BH_4) (6R)-5,6,7,8-tetrahydro-L-biopterin; ITU) isothiourea; 1400W) N-[3-(aminomethyl)-benzyl]-acetamidine; AG) aminoguanidine; 2-AP) 2-aminopicoline; 7-NI) 7-nitroindazole; L-NA) N^{ω}-nitro-L-arginine; L-NMMA) N^{ω}-monomethyl-L-arginine; PA) N^{ω}-propyl-L-arginine; cPA) N^{ω}-cyclopropyl-L-arginine; L-NIO) N^5-iminoethyl-L-ornithine; L-VNIO) L-N^5-(1-iminobuten-3-yl)-L-ornithine; L-NIL) N^6-iminoethyl-L-lysine; L-TC) L-thiocitrulline; S-methyl-TC) S-methyl-L-thiocitrulline; 2-ADT) 2-amino-5,6-dihydro-4*H*-1,3-thiazine.

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0006-2979/05/7001-0008

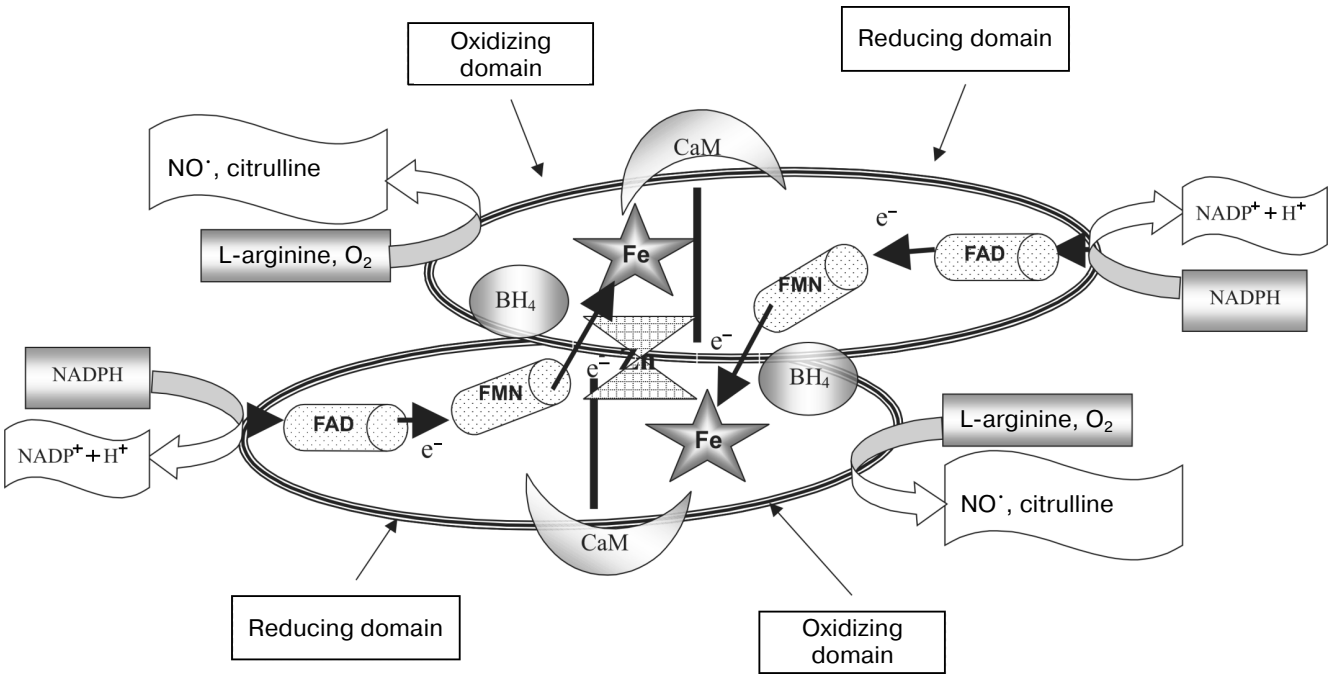


Fig. 1. Scheme of the NOS structure. The homodimeric composition of the enzyme is shown, with "tail-head" contact between the monomers, as well as its domain structure, cofactors, and prosthetic groups. The electron is carried from the reductase domain of one monomer to the oxidase domain of the other monomer under the influence of calmodulin (CaM). CaM is a labile cofactor of constitutive NOS (nNOS and eNOS). CaM produces a tight complex with iNOS, which is really a tetramer not dissociating at low content of Ca^{2+}; therefore, changes in the Ca^{2+} content do not affect the enzyme activity. Peptides (including cysteines which produce a tetracoordinated Zn–thiolate complex) involved in the conformational "lock" between the monomers are mainly located in the oxidase domains.

electrons from NADPH to the oxidase domain which contains the heme (the oxidizing center) and the sites which bind BH_4, L-arginine, and Zn^{2+} (Fig. 1). It should be emphasized that L-arginine, BH_4, and the heme are cooperatively involved in generation of the active enzyme [15]. Therefore, substitution of the substrate by inhibitor directly prevents not only the NO· synthesis but in many cases formation of the active tertiary structure of NOS.

Dimerization. NOS activity is mainly determined by its quaternary structure. The enzyme oxidizes L-arginine and synthesizes NO· only as a homodimeric complex, and an intact cell usually contains ~50% of NOS polypeptides as homodimers [16]. L-Arginine is more effective than BH_4 in promoting the association of the neuronal NOS (nNOS) subunits, but this is the opposite in the case of iNOS. In total, in the presence of the above-mentioned cofactors and substrate, the eNOS dimer of NOS isoforms was the most resistant to dissociation and proteolysis (eNOS >> nNOS > iNOS) [17]. Unexpectedly, L-arginine can bind to the BH_4-binding site of eNOS [18], and this seemed to determine the resistance of eNOS under cofactor shortage.

Proteolysis. Although NOSs are long-living proteins (for example, in human embryo kidney cells HEK-293 half of nNOS molecules are subjected to proteolysis and replaced by new molecules during ~20 h [19]), their resistance to proteolysis seems to considerably contribute to the post-translational regulation of the enzyme activity. In the case of heme loss and shortage of BH_4 and/or L-arginine, the enzyme resistance to proteolysis decreases, but thus seems to be prevented by an allosteric regulator of NOS, the heat shock protein Hsp90 [20].

NOS-associated proteins. The list of NOS protein regulators is rapidly increasing. These proteins influence the enzyme location, its affinity for cofactors (CaM and BH_4), regulate the covalent modification and dimerization of NOS, and, as a result, influence the enzyme activity [13, 21]. Up to now, the inhibitor of trypsin-like serine proteinases aprotinin is the only protein inhibitor of nNOS and iNOS, which can compete with L-arginine [22].

NOS superoxide synthase. In the conjugated reaction NADPH oxidation is accompanied by synthesis of NO· and L-citrulline, but in the so-called uncoupled reaction, depending on the substrate shortage ([L-arginine] < 10 μM and/or [BH_4] < 1 μM), NOS, in addition to NO·, produces reactive oxygen species (ROS)—superoxide anion radical ($O_2^{\cdot-}$) and hydrogen peroxide [23, 24]:

$$\begin{array}{l} \text{NADPH} \quad \text{FAD} \qquad\qquad\qquad\qquad\quad \text{Fe}^{3+} \\ \downarrow \quad \rightarrow \downarrow \rightarrow \text{FADH}\cdot/\text{FMNH}\cdot \rightarrow \downarrow \\ \text{NADP}^{+} \quad \text{FADH}_2 \qquad\qquad\qquad\quad \text{Fe}^{2+} + \text{O}_2 \rightarrow \\ \qquad\qquad\qquad\qquad\qquad\qquad \rightarrow [\text{Fe}^{2+}\text{–O}_2] \rightarrow \text{Fe}^{3+} + \text{O}_2^{\cdot-}. \end{array}$$

This phenomenon is important because, first, ROS initiate other signaling pathways different from those which involve NO˙ and, second, ROS together with NO˙ form a new highly active chemical particle—peroxynitrite ($ONOO^-$).

In the absence of L-arginine, nNOS is the most active superoxide synthase [23]. Under these conditions, it oxidizes NADPH an order of magnitude faster (~2 μmol/min per mg protein) than two other isoforms and produces a greater amount of O_2^-, respectively. The superoxide synthase activity of nNOS is suppressed by L-arginine [24]. The production of O_2^- under the influence of iNOS is virtually independent of the L-arginine concentration. This may be because the reductase domain of iNOS is a source of O_2^- [25]. The chaperon Hsp90 is important for maintaining the balance between syntheses of ROS and NO˙ in NOS [26].

The content of NO˙ can be determined by both the above-described mechanisms and chemical reactions of the radical with intracellular NO˙ inactivators—hemoglobin [27], cytochrome *c* oxidase [28], O_2^- and HOCl [29]. Effects of substrate-like NOS inhibitors on these reactions are virtually unknown. It seems that data on interactions of NOS inhibitors with deposited, transporting, and effector NO˙ forms will be especially interesting [30].

Structure of the NOS oxidase domain. This domain contains the four most important structures of the enzyme: the oxidizing center containing the heme with pentacoordinated iron, the L-arginine-binding site, the BH_4-accepting cavity, and the Zn–thiolate complex (Fig. 1) [18]. The main role in the binding of L-arginine is played by two conservative amino acids. In that nearest to the heme cavity (proximal) the proton of the amine group of the L-arginine guanidine moiety is accepted by the carbonyl group of tryptophan. The other proton of the same amine group and the proton at N^5 are bound in the distal cavity by the carbonyl groups of glutamic acid. Figure 2 shows the arrangement of a NOS inhibitor N^{ω}-propyl-L-arginine (PA) inside the guanidine-binding cavity of NOS. The carbonyl groups of Trp and Glu are involved in the binding of PA in the same manner as of L-arginine. One of the axial bonds of 5- or 6-coordinated Fe^{3+} is occupied by cysteine residue from the NOS polypeptide, whereas another bond can be occupied by an inhibitor, and this significantly influences the inhibitory activity of such a compound [31].

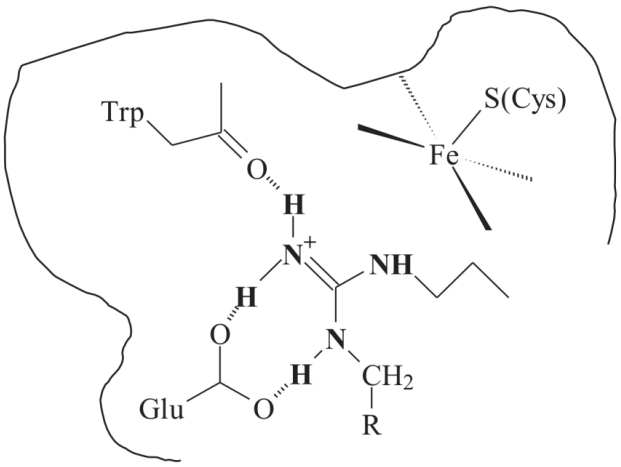


Fig. 2. Scheme of supposed interaction of N^{ω}-propyl-L-arginine (a substrate-like NOS inhibitor) with the NOS catalytic center where L-arginine is bound and oxidized [31]. Two amino acids of the acceptor pocket, Glu and Trp, play the main role in binding the substrate or its analogs. L-Arginine-like inhibitors can differently interact with the oxidizing pocket containing the heme with 5- or 6-coordinated iron: displacing the sixth ligand (if it exists) without formation of a bond or producing the sixth coordinated bond (e.g., L-thiocitrulline). R = HOOC-CH(NH_2)-(CH_2)-.

Spectral and inhibitory analyses of nNOS, iNOS, and eNOS revealed specificity in the ratio of the substrate cavity size in the isoforms: nNOS > iNOS > eNOS [32, 33]. Similar topological features are also found in the distal and proximal (relatively to the heme) "pockets" which accept the guanidine group protons. The site nearest to the heme that is responsible for the binding of an amine group, which is a source of N for NO˙ can be occupied by considerably larger groups than -NH_2 or =NOH. On the contrary, the distal NH_2-binding site has no place for such groups. These features of the substrate-binding center topology are also observed in isoforms. Thus, iNOS poorly binds N-alkyl groups larger than ethyl in the near-heme "pocket" and cannot accept -SR in the distal NH_2-binding cavity, whereas the binding by nNOS is more efficient [31].

Although a conservative similarity of NOS polypeptides and, in particular, of the oxidase domains makes difficult the search for selective inhibitors, minor differences in the primary and quaternary structures of NOS can be determining due to the high cooperativity in the interaction of the substrate cavity, heme, BH_4, and also peptide motifs involved in the enzyme dimerization, covalent modification, and location.

ACTIVITIES AND ACTION MECHANISMS OF L-ARGININE-LIKE INHIBITORS OF NO˙ SYNTHESIS

After the discovery of enzymatic origin of NO˙, hundreds of compounds were studied as NOS inhibitors using isolated enzyme of various purity as a model. However, these important and useful data were obtained in the absence of conventional standards for conditions of such studies (the species as the enzyme source, the isolation methods, the reaction conditions, etc.) [34]. The NOS-inhibiting activity of compounds under study is usually characterized by stability of the inhibitor–enzyme com-

plex described by parameters K_i and IC_{50} (Table 1). By values of these parameters, the compounds will be divided into four groups with K_i (IC_{50}) > 100, 10-100, <10, and <1 μM, respectively. Data on selective inhibition of particular NOS isoforms will be presented as ratios K_i^{eNOS}/K_i^{iNOS} (e/i) and K_i^{nNOS}/K_i^{iNOS} (n/i) (or as the corresponding ratios of IC_{50}). Four groups of inhibitors will be also presented based on values of the "selectivity" parameter: e/i and n/i <1 or >1; e/i and n/i <0.1 or >10; e/i and n/i <0.01 or >100; and e/i and n/i <0.001 or >1000.

Amino acids and their derivatives containing the guanidine group. N^{ω}-Nitro-L-arginine (L-NA), N^{ω}-monomethyl-L-arginine (L-NMMA), and aminoguanidine (AG) were among the first compounds containing different substituents in the guanidine group (Table 1). The selectivity of L-NA to constitutive NOS is determined by its slow binding and an extremely slow separating from the enzymes in the presence of the substrate [43]. The methyl ester of this inhibitor (L-NAME) suppressed the synthesis of $O_2^{\cdot-}$ in nNOS-transfected HEK-293 L-arginine-free cells [44] due to inhibition of the heme iron reduction [45]. Similarly to L-NA, L-NMMA is a moderate inhibitor of all NOS, lacks pronounced selectivity (K_i < 10 μM, n/i and e/i <1), and acts as a "suicidal" inhibitor. This compound is hydroxylated by NOS as a pseudosubstrate, similarly to L-arginine. However, products of the next stage (CH_3NO^+, CH_2O, etc.) covalently modify the enzyme, with probable removal of the heme [46]. In the absence of BH_4, L-NMMA did not suppress $O_2^{\cdot-}$ synthesis in the enzymatic model of eNOS [47]. L-NMMA also stimulated proteolysis of nNOS in human embryo kidney cells (HEK-293) [19].

The inhibitory activity of L-arginine derivatives with aliphatic substituents (R = -methyl, -ethyl, -propyl, -butyl) in the N^{ω}-position significantly decreased for iNOS with increase in the substituent size (>4000 μM for R = -butyl) and increased for nNOS [31]. PA, which is a weak inhibitor of iNOS (IC_{50} ~ 950 μM), had a high selectivity to nNOS (n/i < 0.01, or < 0.001 by data of [48]) and a moderate selectivity to eNOS (e/i < 0.1) (Table 2). cPA was a strong inhibitor of m-iNOS (K_i ~ 8 μM) [49]. Introduction of an unsaturated bond into the substituent (R = -allyl, -propargyl) decreased the inhibitor activity for nNOS and increased it for iNOS and eNOS. This seems to be caused by an inactivating effect on iNOS of inhibitors containing an unsaturated bond [48]. However, this was associated with a virtual loss of selectivity (n/i < 1 and e/i > 1 for N^{ω}-propargyl-L-arginine).

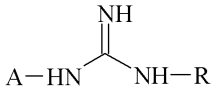
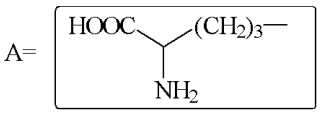
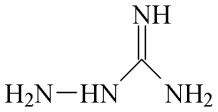
Some natural amino acids, which contain the guanidine group and display NOS-inhibiting activity, can also be used for chemical design of more effective inhibitors. The amino acid of plant origin L-canavanine (with the δ-methylene group of L-arginine substituted by O) was a poor inhibitor of $NO^{\cdot}$ synthesis (IC_{50}^{nNOS} ~ 130 μM) and lacked pronounced selectivity. This could be caused by impossibility of hydrogen bond generation with H at N^5 because the preferential L-canavanine tautomer does not have the necessary hydrogen atom [31]. However, physiological experiments show the iNOS-specific activity of this amino acid [50]. Recent repeated studies on agmatine (the decarboxylated arginine, 1-amino-4-guanidinobutane (AG)) revealed its new NOS-inhibiting properties. Agmatine increased oxidation of NADPH and production of H_2O_2 by nNOS that resulted in the enzyme inactivation. During the initial reversible period of the inhibition, its affinity for nNOS was characterized by K_i ~ 30 μM [51].

Similarly to other guanidine derivatives, AG irreversibly inactivates all NOS isoforms (Table 2). They accelerate the self-inactivation of nNOS under conditions of the substrate-independent oxidation of NADPH [38]. An amino acid analog of AG (N^{ω}-amino-L-arginine) also irreversibly inactivates NOS both *in vitro* and *in vivo*, probably by removal of the heme from the polypeptide or by covalent binding to the heme [52]. Substitution of the amine group by the alkyl in AG noticeably decreases the inhibitory activity (for N-methylguanidine IC_{50}^{iNOS} = 100 μM) [38]. 2-Ethyl-1-aminoguanidine was the strongest inhibitor of iNOS (IC_{50}^{iNOS} = 1 μM) as compared to methyl-, hydroxyl-, and phenyl-substituted guanidines in the (1-) and (2-) positions. Unlike AG, this compound inhibited iNOS more selectively (n/i and e/i > 10).

With L-NA as the basic structure, various N^{ω}-nitro-L/D-arginine derivatives were synthesized as dipeptides containing D- and L-isomers of phenylalanine (Table 1) [39]. And among poorly effective iNOS inhibitors (K_i^{iNOS} > 1000 μM), compounds were found with a unique selectivity to nNOS and eNOS. In particular, the selectivity parameters of methyl ester D-Phe-D-NA-OMe were <0.001 for n/i and <0.01 for e/i. Note that D-NA- and D/L-Phe-containing dipeptides were noncompetitive inhibitors of iNOS, but competitively inhibited nNOS and eNOS. These findings suggest the presence of specific binding sites in iNOS, which accept the amino acid radical of unnatural geometry outside the guanidine-binding cavity.

Substitution in dipeptides of the carboxyl group by the amide group resulted in active and selective inhibitors [40]. One of the least active iNOS-inhibitors, L-Lys-D-NA-NH_2 (K_i^{iNOS} > 1000 μM), was selective to all three isoforms (n/i < 0.001, e/i < 0.1, n/e < 0.01). Reduction of the amide group to amine in these stereoselective compounds resulted in a pseudopeptide (4*S*)-N-[4-amino-5-(aminoethyl)-aminopentyl]-N′-nitroguanidine which had a moderate iNOS-inhibiting activity (K_i^{iNOS} > 10 μM) and a high selectivity to nNOS (n/i < 0.01 and n/e < 0.001) [53]. Synthesis in this direction gave a new active compound, N-(4*S*)-{4-amino-5-[2-(2-aminoethyl)phenylamino]-pentyl}-N′-nitroguanidine (K_i^{iNOS} = 3.5 μM, n/e < 0.001, n/i ~ 0.01) [54].

Table 1. Derivatives of L-arginine and guanidine[a]

Structure	Name of compound (code)	K_i^{iNOS}, μM	Selectivity[b]		References
			n/i[c]	e/i	
	N^{ω}-nitro-	3[d]	0.09	0.1	[14]
	L-arginine	2[e]	0.02	0.05	[35]
	(L-NA)	13[f]	0.2	0.09	[36]
		0.7[g]	0.02	0.04	[37]
	N^{ω}-monomethyl-	7	0.7	0.5	[14]
R = -NO_2; -CH_3; -C_3H_7	L-arginine	5[h]	0.2	0.2	[31]
	(L-NMMA)	1.3	1	0.3	[35]
A= 		1	1	0.5	[37]
	N^{ω}-propyl-	100	0.004	0.05	[31]
	L-arginine (PLA)	25	0.02	0.08	[35]
	Amino-	30	6	10	[14]
	guanidine	30	12	9	[35]
	(AG)	5[i]	8	50	[38]
D-Phe-D-NA-OCH_3	D-phenylalaninyl-N^{ω}-nitro-D-arginine methyl ester	3600[j]	<0.001	<0.001	[39]
L-Lys-D-NA-NH_2	L-lysyl-N^{ω}-nitro-D-arginine amide	4700[j]	<0.001	<0.1	[40]

[a] All data in this and the following tables are approximated for the purpose of the present review and, therefore, may not be used instead of the exact values presented in the original papers. IC_{50} is the inhibitor concentration which 50% suppresses the NOS activity at the given L-arginine concentration. On assumption of competitiveness and steady state of the reaction, the K_i value is usually calculated by the equation: $1/v = 1/V_{max} + (1 + [I]/K_i)K_m^{Arg}/(V_{max}[Arg])$, where [I] and [Arg] are concentrations of the inhibitor and L-arginine, respectively; K_m^{Arg} is the Michaelis constant.

[b] The prevalent inhibition of one NOS isoform compared to another is expressed by ratio of the corresponding values which determine the inhibitor activity (IC_{50} or K_i): $n/i = IC_{50}^{nNOS}/IC_{50}^{iNOS}$, $e/i = IC_{50}^{eNOS}/IC_{50}^{iNOS}$.

[c] The species-specific source of NOS indicated as m-, r-, b-, h- are mouse, rat, bovine, and human, respectively.

[d] IC_{50}; [Arg] = 30 μM, h-NOS.

[e] The K_i value is calculated from data of this paper ([Arg] = 0.1 μM, K_m^{Arg} ~ 6, 3, and 3 μM for h-iNOS, h-nNOS, and h-eNOS, respectively) and from the ratio $IC_{50} = K_i(1 + [Arg]/K_m^{Arg})$ [31, 41, 42].

[f] m-iNOS, r-nNOS, b-eNOS.

[g] h-NOS.

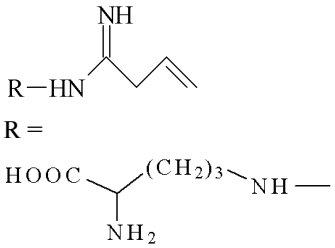
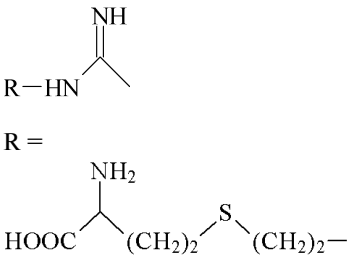
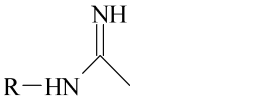
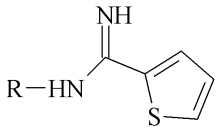
[h] The K_i value is calculated from data of this paper (m-iNOS ([Arg] = 100 μM, K_m^{Arg} = 12.5), r-nNOS ([Arg] = 20 μM, K_m^{Arg} = 1.8 μM), b-eNOS ([Arg] = 30 μM, K_m^{Arg} = 3.6 μM)), see [e].

[i] IC_{50}; [Arg] = 120 μM, m-iNOS, b-nNOS, b-eNOS.

[j] m-iNOS, b-nNOS, b-eNOS.

The amino acid moiety is shown to be not necessary for substrate-like inhibitors [55]. nNOS-inhibiting activity was found in a known vasorelaxation drug, guanabenz (1-(-2,6-dichlorobenzylidenamino)-guanidine), which is an agonist of central α_2-adrenoreceptors. In the cytosol of HEK-293 cells, this preparation reversibly inhibited at first the enzyme activity (K_i^{nNOS} = 1 μM) and then irreversibly inactivated nNOS. The lifetime of nNOS in the

Table 2. Inhibitors containing the amidine group

Structure	Name of compound	K_i^{iNOS}, μM	Selectivity		References
			n/i	e/i	
	L-N^5-(1-iminobuten-3-yl)-L-ornithine (L-VNIO)	60[a]	0.002	<1	[56]
	S-{2-[(1-iminoethyl)-amino]-ethyl}-L-homocysteine (GW274150)	1.4[b]	300	100	[57]
	N-[3-(aminomethyl)-benzyl]-acetamidine (1400W)	0.2[c] 0.14[d]	>10 >10	>1000 ~500	[14] [37]
	N-{4-[2-([(3-chlorophenyl)-methyl]amino)-ethyl]phenyl}-2-thiophene-carboxamidine (AR-R17477)	5[e] 0.3[c]	0.007 <1	<1 >1	[12] [14]

[a] m-iNOS, b-nNOS, and b-eNOS.
[b] K_i is calculated (see Table 1[e]) from data of this paper ([Arg] = 30 μM, K_m^{Arg} ~ 7, 6, 4 μM for h-iNOS, h-nNOS, and h-eNOS, respectively).
[c] IC_{50}, [Arg] = 30 μM, h-NOS.
[d] h-NOS.
[e] IC_{50}, m-iNOS, r-nNOS, h-eNOS.

cell culture decreased from 20 to 10 h in the presence of guanabenz [19]. Thus, effective NOS inhibitors have been found among compounds with a highly basic guanidine-like group. It should be noted that the strength of binding with the enzymes significantly depends on the other moiety of the inhibitor molecule, which also determines its selectivity for NOS isoforms.

Inhibitors containing the amidine group. According to a model of the L-arginine binding cavity of NOS (Fig. 2), modification or complete substitution of the amine group oxidized to NO$^{\bullet}$ does not always significantly affect the interaction of a substrate-like inhibitor with the enzyme. An increase in the substituent ($R = C_nH_{2n+1}$) in N^5-(1-iminoalkyl)-L-ornithine molecules decreased the activities of inhibitors to all NOS isoforms, whereas the affinity of N^{ω}-alkyl-L-arginines for nNOS increased [31]. The introduction of an unsaturated bond into the substituent with $n = 3$, similarly to the case of N^{ω}-alkyl-arginines, significantly increased the selectivity of the resulting compound L-VNIO for nNOS ($IC_{50}^{iNOS} = 200$ μM; n/i < 0.01, e/i < 1) (Table 2). This selectivity (also observed with respect to eNOS) seems to be mainly caused by the inactivating mechanism of the L-VNIO interaction with the enzyme. Moreover, L-VNIO suppressed the NADPH oxidase activity of nNOS [56]. L-NIL was the most active inhibitor of iNOS among amino acids with the amidine group, and, unlike L-ornithine derivatives, this compound had a moderate selectivity for iNOS (n/i and e/i > 10) [14, 58]. Thus, the effect of aliphatic substituents in the carboxamidine group on the inhibitory activity of iNOS was similar to the effect of introduction of similar substituents into the guanidine group.

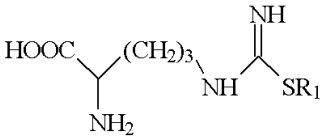
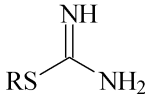
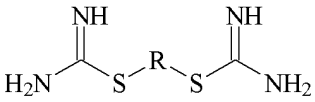
A series of stereospecific NOS inhibitors (L-NIL analogs) has been produced on the base of L-cysteine and L-homocysteine (Table 2). Among the derivatives different in the S-atom substituents (O, SO, or SO_2), L-isomers displayed higher iNOS-inhibiting activity (IC_{50} = 0.7-1.6 μM) than D-isomers. Two compounds of this series containing 4,4-dioxo-L-cysteinyl (GW273629) and L-homocysteinyl (GW274150) had a high selectivity to iNOS [57].

The discovery of the iNOS-selective inhibitor N-[3-(aminomethyl)-benzyl]-acetamidine (1400W) seems to be the most interesting result in studies on non-amino acid inhibitors containing the amidine group [59]. Their activities and specificities (Table 2) are more likely determined by the two-stage chemical interaction of the inhibitor with iNOS. Unlike the fast and reversible interaction of 1400W with constitutive NOS, the second stage of the inhibitor reaction with iNOS is slow and virtually irreversible. Note that nNOS-selective inhibitors occurred among other analogs of this fundamental structure (arylamidines) [37]. In particular, this is exemplified by a compound which is different from 1400W in a single methylene group, N-[3-(aminomethyl)-phenyl]-acetamidine ($K_i^{iNOS} = 2$ μM, n/i < 0.1, e/i > 1). However, N-[3-(aminomethyl)-phenyl]-acetamidine had no *in vivo* effect on nNOS activity in rat brain. Inclusion of the thiomethyl group into the acetamidine group resulted in the most active compound of this class, (N-[3-(aminomethyl)-phenyl]-thiomethyl-acetamidine, with respect to both iNOS and nNOS (K_i^{iNOS} and K_i^{nNOS} = 0.01 μM). A compound with the fluoromethyl substituent in the acetamidine group was also a powerful inhibitor of nNOS. Substitution of the acetamidine group by the arylamidine group also significantly increased the nNOS-inhibiting activities of resulting compounds ($K_i^{nNOS} = 6$ and 9 nM for 2-furanyl- and 2-thienyl-derivatives, respectively). Highly active iNOS inhibitors were also found among other 2-thienylcarboxamidines, e.g., AR-R17477 (Table 2). Although the authors considered the selectivity of these derivatives to be insufficient, the thienyl substituent manifested itself as a very promising pharmacophor [12]. Combining the thiophencarboxamidine group with the active moiety of vitamin E in the same molecule resulted in a highly selective nNOS inhibitor ($K_i^{r\text{-}nNOS} \sim 0.9$ μM; $K_i^{b\text{-}eNOS} \sim 110$ μM; $K_i^{m\text{-}iNOS} >$ 300 μM) and antioxidant (BN80933) with a pronounced anti-ischemic effect [36]. Thus, the decrease in basicity of the guanidine-like group, which determines the inhibitor binding to the enzyme, by a complete substitution of one of the amine groups did not weaken the inhibitory activity of the resulting compounds. Moreover, modification of the carboxamidine residue with aryl radicals yielded extremely active compounds.

L-Thiocitrulline and its isothiuronium analogs. L-Citrulline analogs are numerous heterosubstituted compounds with oxygen or sulfur atoms instead of one of the guanidine nitrogens. L-Citrulline itself is a very weak inhibitor of NOS [31]. However, its analog L-thiocitrulline (L-TC) is a strong inhibitor, but it inhibits nNOS about 50-fold stronger than iNOS (Table 3). The firmness of L-TC binding with the enzyme is, in particular, determined by the binding between the heme iron and the thiol group of the inhibitor as with the sixth ligand, and this is confirmed by changes in spectral characteristics (type II differential spectrum). This seems also to determine the inhibition by L-TC of the NADPH oxidase activity of nNOS [60] but not of eNOS [61].

Introduction of an aliphatic radical into the thiol group of L-TC significantly increased the binding of such compounds with the enzyme. But their selectivities were lost [42]. With increase in the substituent (C_nH_{2n+1}) size to $n = 4$, the inhibitory activity was nearly 50-fold decreased. Unlike L-TC, the binding of aliphatic derivatives with the enzyme was characterized by the I type spectral change that represented only the displacement of the sixth ligand from the hexacoordinated iron of the heme without binding to it [25]. These groups of inhibitors, unlike N^{ω}-alkyl-L-arginines, were not stereoselective to the enzyme. S-Me-D-TC was a weak inhibitor of iNOS and nNOS ($IC_{50} \sim 470$ and 160 μM, respective-

Table 3. S-Substituted derivatives of citrulline and thiourea

Structure	Name of compound (code)	K_i^{iNOS}, μM	Selectivity		References
			n/i	e/i	
 R_1 = -H, -CH_3	L-thiocitrulline (L-TC)	4[a]	0.02		[60]
	S-methyl-L-thiocitrulline (S-Me-L-TC)	0.8[b]	<0.1		[42]
	S-2-aminoethyl-ITU	6[c]	>1	>1	[35]
	S-ethyl-ITU	0.02[d]	>1	>1	[61]
		0.1[c]	>1	1	[35]
		0.02	>10	>10	[62]
R = H_2N-$(CH_2)_2$- , -CH_2CH_3, i-(C_3H_7)- , 3-(CH_3O)-C_6H_4-$(CH_2)_2$-	S-isopropyl-ITU	0.01[d]	>10	>10	
	S-3-methoxy-phenethyl-ITU	0.2[d]	>1	>10	[61]
 R = -$(CH_2)_2$-(1,4)-C_6H_4-$(CH_2)_2$-	S,S′-[1,4-phenylene-bis(1,2-ethandiyl)]-bis-ITU	0.007[d]	>1	>10	

[a] r-NOS.
[b] r-NOS.
[c] The K_i is calculated (see Table 1[e]) from data of this paper ([Arg] = 0.1 μM, K_m^{Arg} ~ 6, 3, and 3 μM for h-iNOS, h-nNOS, and h-eNOS, respectively).
[d] h-NOS.

ly). Similar data were also obtained for N^5-2-nitrophenyl-substituted ornithines. N^5-(5-methyl-2-nitrophenyl)-D-ornithine inhibited r-nNOS only about 30-fold weaker than the L-isomer (IC_{50} = 2.5 μM) [63]. S-Alkyl-L-thiocitrullines were different in the activity and selectivity to the enzymes from different sources (human and rat cells) [64]. Inhibitory activities of isothiourea (ITU) non-amino acid derivatives to iNOS from mouse and human cells were also different [61].

A number of compounds base on L-TC were also studied, in particular, S-Me-L-TC-L-phenylalanine, which is a very strong and selective inhibitor of iNOS (IC_{50} = 0.65 μM, n/i ~ 60) [65]. Substitution in L-citrulline or L-TC of the second amine group by the alkyl group virtually abolished the inhibitory activity of these compounds. Thus, for N^5-acyl-L-ornithines and N^5-thioacyl-L-ornithines $IC_{50} \geq 1000$ μM. This indicated a poor affinity of heteroatoms S= and O= for the catalytic center in the presence of C^6-alkyl group [31].

Among isothiourea (ITU) derivatives, ethyl-ITU and isopropyl-ITU were the most active inhibitors of iNOS, but their selectivity to this isoform was low both *in vitro* (Table 3) and *in vivo* [66]. All S-alkyl-ITU were competitive and reversible inhibitors and interacted with the heme similarly to L-arginine [42, 61, 62]. S-Ethyl-ITU promoted the dimerization of iNOS more strongly than L-arginine itself [67]. S-Aminoethyl-ITU and AG also inhibited expression of the iNOS protein in mouse macrophages and rat lungs [68]. These compounds effectively inhibited the *in vivo* LPS-induced NO˙ synthesis [7]. S-Phenylalkyl derivatives of ITU compounds did not display a high selectivity, except S-3-methoxyphenethyl-ITU (e/i ~ 20) [69]. N-Phenyl derivatives of ITU, in particular, N-phenyl-ITU, which had poor selectivity to

Table 4. The main mechanisms of NOS inhibition by substrate-like compounds

NOS	Fast reversible binding	Slow binding and dissociation	Enzyme inactivation
iNOS	L-NA (m*) [43, 77] L-cPA (m) [48] L-VNIO (m) [56] Methylguanidine (m) [38] L-Canavanine [31] 2-AP (h) [35]	1400W (h) [59]	L-NMMA (m) [43] N^{ω}-amino-L-arginine (m), AG (m) [38]; L-NIO [78], N^{ω}-allyl-L-arginine (m) [48] L-NIL (h), GW274150 (h), GW273629 (h) [14]
nNOS	Methylguanidine (b) [38] 1400W (h) [59] 2-AP (h) [35]; L-TC (r) [60]	L-NA (b) [43, 77] S-Me-TC (h) [64]	N^{ω}-amino-L-arginine (b) [38], L-NMMA [43] L-NIO, L-VNIO (r) [56]
eNOS	NMMA (b) [43] Methylguanidine, 1,1-dimethylguanidine (b) [38] 1400W (h) [59]; 2-AP (h) [35]	L-NA (b) [43, 77]	N^{ω}-amino-L-arginine (m), AG (m) [38] L-VNIO (b) [56]

* In parentheses species are presented which were used as sources of the enzyme used in studies on the interaction mechanism of the inhibitor with NOS: (b) bovine, (m) mouse, (r) rat, (h) human.

nNOS (K_i^{iNOS} ~ 0.9 μM, n/i ~ 0.1 and e/i ~ 0.5), inhibited nNOS more actively. Among other derivatives of S-ethyl-N-phenyl-ITU (SENPITU) there are considerably less active toward iNOS but more selective to nNOS compounds. For 2- and 4-trifluoromethyl derivatives K_i ~ 20 μM, n/i ~ 0.06, and e/i ~ 1 and K_i ~ 40 μM, n/i ~ 0.01, and e/i ~ 0.3, respectively. But the latter compound failed to inhibit nNOS in tissue culture [70].

An attempt to prepare more active isothiuronium derivatives by introduction of the N-amine group similarly to aminoguanidine was unsuccessful. The resulting compounds were 100 and more times less active than the corresponding S-alkyl-ITU and without pronounced selectivity. Like aminoguanidine, they interacted with NOS via inactivation and accelerated the nNOS self-inactivation in the absence of substrate [38]. It should be noted that S-aminoethyl-ITU also suppressed the *in vivo* translation of iNOS and accelerated its degradation in cell culture RAW 264.7 [71]. Moreover, under these conditions S-aminoalkyl-ITU spontaneously rearranged into mercaptoalkyl guanidines [72], which also inhibited iNOS [73] and, moreover, effectively intercepted $ONOO^-$, a particle playing a pathogenic role in some diseases [74].

Compounds containing two isothiuronium groups were more active [61]. Change of the *p*-position of substituents in 1,4-PBITU (Table 3) by the *m*-position was associated with a slight decrease in the inhibitory activity of S,S′-[1,3-phenylene-bis-(1,2-ethandiyl)]-bis-ITU (1,3-PBITU) to iNOS and a significant decrease to eNOS, as compared to 1,4-PBITU; thus, this compound seemed to be highly selective (K_i^{iNOS} ~ 0.05 μM, n/i > 200, e/i ~ 5).

Crystallographic studies on bis-ITU (and selenium analogs) complexes with eNOS showed that the affinity of inhibitors for this constitutive enzyme was partially caused by interaction of the second ureido group with propionate of the heme pyrrole ring D [75]. An increase in the size of the group binding the two isothiuronium residues to C_{12-14} also resulted in active NOS inhibitors. This was probably caused by involvement in the contact with the inhibitor of new peptide motifs outside the catalytic center and the substrate availability channel because, unlike simple S-alkyl-ITU, these bis-ITU inhibited the electron transfer in the reductase domain of NOS [76]. Unfortunately, toxicity of such bis-isothiuronium derivatives and poor permeability across cell membranes considerably prevent their use in practice [61].

Thus, the inhibitory activity of the above-described compounds and their selectivity are directly determined by mechanism of the enzyme interaction with the inhibitor. These mechanisms for the most often used compounds are summarized in Table 4. The strongest and most selective inhibitors are characterized either by kinetics of binding to the inhibitor (slow binding and slow dissociation) or by the inactivation type of inhibition ("suicidal" inhibitors). These "suicidal" inhibitors (L-NMMA, L-NIL, aminomethyl-ITU) also increased proteolysis of NOS, as differentiated from reversible inhibitors (L-NA, 7-NI, ethyl-ITU), which promote the resistance of the NOS dimer [19, 20].

CYCLIC ANALOGS OF SUBSTRATE-LIKE NOS INHIBITORS

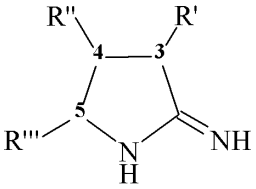
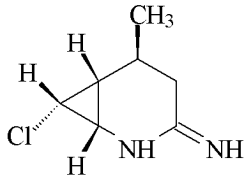
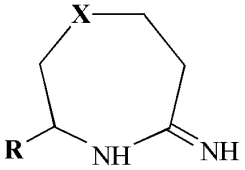
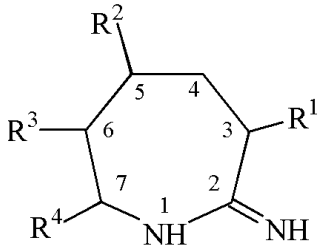
Inclusion of the guanidine-like group into a cyclic structure opens a large field for chemical construction of

NOS inhibitors recognizable by the target enzyme due to likeness to L-arginine.

1*H*-Pyrazole-1-carboxamidines. 1*H*-Pyrazole-1-carboxamidines are competitive NOS inhibitors containing one of guanidine group nitrogens in the cycle [79, 80]. N^G-substituted derivatives, such as 1*H*-pyrazole-N-(1,2-diaminoethyl)-1-carboxamidine and 1*H*-pyrazole-N-(3-aminoethylanilino)-1-carboxamidine, displayed the most pronounced selectivity (K_i^{iNOS} ~ 10 μM, n/i ~ 0.1, e/i ≥ 10).

Cyclic amidines. Compounds containing the amidine group, mainly heterocyclic imines and imidazoles, are the best studied cyclic inhibitors of NOS. Modification of the pyrrolidine ring by aliphatic substituents in the fifth position near the heterocyclic nitrogen sharply increased the activity and selectivity of new compounds (Table 5). The iNOS-inhibiting activity was increased by two order of magnitude compared to the parent compound (K_i^{iNOS} < 1 μM). Separation of racemates indicated that (+)-*cis*-5-pentyl-isomers were nearly an order of magnitude more selective to iNOS than to eNOS (K_i ~ 0.1 μM, n/i ~ 10, e/i > 100) [81]. This finding suggested the opening of new stereospecific receptors of iNOS. The recently described new iNOS inhibitor (3S,4S,5R)-3-hydroxy-4-methyl-5-pentyl-pyrrolidine-2-imine was still more selective (e/i), and this suggested that the search for stereoselective iNOS inhibitors among iminopyrrolidines should be promising [82]. These compounds were also active in the *in vivo* model of endotoxic shock. The bicyclic amidine ONO-1714 (Table 5) displayed an extremely high

Table 5. Heterocyclic compounds with the amidine group in the cycle

Structure	Name of compound (code)	K_i^{iNOS}, μM	Selectivity		References
			n/i	e/i	
	(+)-4-methyl-*cis*-5-pentyl-pyrrolidine-2-imine	0.07[a]	10	400	[81]
	(3S,4S,5R)-3-hydroxy-4-methyl-5-pentyl-pyrrolidine-2-imine	0.15[a]	>10	>1000	[82]
	(1*S*,5*S*,6*S*,7*R*)-3-imino-5-methyl-7-chloro-2-azabicyclo[4.1.0]heptane (ONO-1714)	0.002[b]		10	[83]
	2-imino-7-butyl-5-oxo-homopiperidine (R = -$(CH_2)_3CH_3$, X = O)	0.5[a]	10	300	[84]
	Hexahydro-7-propyl-1*H*-azepine-2-imine	0.03[c]	>1	>10	[85][a]
	Hexahydro-7-(2-ethylbutyl)-1*H*-azepine-2-imine	0.4	>1	200	

[a] K_i is calculated (see Table 1[e]) from data of this paper ([Arg] = 30 μM, K_m^{Arg} = 7, 6, 4 μM for h-iNOS, h-nNOS, and h-eNOS, respectively).
[b] h-NOS.
[c] The experimental value of K_i is 0.09 μM.

inhibitory activity to h-iNOS not only *in vitro* (K_i ~ 0.002 μM, e/i ~ 10) but also in the test of suppression of the LPS-induced nitrite production in mice (ID_{50} ~ 0.01 mg/kg) [83]. Modification of seven-member cyclic amidines in the 7th position resulted in more selective iNOS inhibitors (Table 5). The selectivity was determined by hydrophobic substituents (-butene, -butyl, -2-ethylbutyl, -propene), whereas introduction of the polar nitroethyl group decreased it (e/i ~ 20), although this was associated with nearly an order of magnitude increased iNOS-inhibiting activity of this derivative [84]. Highly active and selective inhibitors (K_i < 1 μM, e/i > 100) were found on introduction of substituents into the 7th position near the heteroatom in the hexahydro-1*H*-azepine-2-imines [85]. Some cyclic amidines, in particular, 2-iminopiperidine [58] and hexahydro-7-butyl-1*H*-azepine-2-imine [85], when tested *in vivo*, also effectively inhibited the LPS-induced production of nitrite.

The search for NOS inhibitors among heterocyclic imines was reasonably continued by synthesis of unsaturated iminopiperidine analogs aminopyridines. 2-Amino-4-methylpyridine was one of the first under study [86]. Later, this inhibitor ($K_i^{\text{h-iNOS, h-nNOS, h-eNOS}}$ = 55, 65, and 98 μM, respectively) was used as a competitive ligand to assess the affinity and selectivity of other NOS inhibitors [35]. 2-Aminopyridines condensed with five- or six-member saturated and unsaturated cycles also were active iNOS inhibitors (IC_{50} < 10 μM). And the derivatives with the unsaturated cycles were more active than the compounds containing the saturated cycles [87]. The iNOS-inhibiting activity was undeterminable in 6-[4-(substituted)-phenyl]-2-aminopyridines. However, these compounds strongly inhibited h-nNOS (IC_{50} < 1 μM) with insignificant selectivity to h-eNOS (e/n > 1) [88].

Imidazoles and nitroindazoles. Imidazole derivatives, which may be considered as cyclic carboxamidine analogs containing both amidine nitrogens in the heterocycle, present a special group of NOS inhibitors. These compounds are characterized by the heme as the main target in their interaction with the enzyme. Imidazoles bind to the enzyme as the sixth ligand of the 6-coordinated iron in the heme, similarly to CO, $NO^{\bullet}$, O_2, CN^-, i.e., by the pattern studied on cytochromes P-450, which are very like NOS [89]. The known imidazoles inhibit NOS reversibly, but, depending on substituent and source of NOS, BH_4 and/or L-arginine compete with them for binding to the catalytic center (Table 6).

Highly effective inhibitors were not obtained among 1,2-substituted imidazoles, although compounds with this structure can be useful for studies on guanidine-, oxygen-, and BH_4-binding sites of NOS [91-94]. The most important specific feature of imidazole and, possibly, its derivatives is the ability to inhibit the nNOS superoxide synthase activity. This seems to be specific for compounds complexing as the sixth ligand with the heme iron atom [47]. It was shown by crystallographic studies that two imidazole molecules can concurrently bind to the m-iNOS oxidase domain: one in the catalytic center as the sixth ligand of the heme iron and another with Glu(371) in the guanidine-binding cavity [95]. This was responsible for the synthesis of guanidine-substituted imidazoles. However, most of the preparations tested had either a low activity or were inactive. Among them, N^{ω}-(2-methylimidazole)-L-arginine was the most active toward iNOS (IC_{50} ~ 140 μM) [96].

Table 6. Roles of L-arginine and BH_4 in the inhibitory activity of imidazole and nitroindazole

Name of compound (code)	K_i, μM (m-iNOS*/ h-iNOS)	Type of inhibition [89]				NADPH oxidase** [90]
		m-iNOS	h-iNOS	b-nNOS	b-eNOS	b-nNOS
Imidazole	48/95	$nc^{(Arg)}$*** $nc^{(BH_4)}$	$c^{(Arg)}$ $c^{(BH_4)}$	$nc^{(Arg)}$ $nc^{(BH_4)}$	$c^{(Arg)}$ $nc^{(BH_4)}$	+
1-Phenyl-imidazole	0.7/38	$nc^{(Arg)}$ $c^{(BH_4)}$	$c^{(Arg)}$ $c^{(BH_4)}$	$nc^{(Arg)}$ $nc^{(BH_4)}$	$c^{(Arg)}$ $c^{(BH_4)}$	+
7-Nitro-indazole [90]	1.6	$nc^{(Arg)}$ $c^{(BH_4)}$		$c^{(Arg)}$ $c^{(BH_4)}$	$nc^{(Arg)}$ $c^{(BH_4)}$	+

* (m-iNOS/h-iNOS) present K_i values for mouse and human iNOS, respectively.

** NADPH oxidase: this column shows the ability of compounds to inhibit the NADPH oxidase activity of NOS. Symbol "+" denotes suppression of the substrate-independent NADPH oxidase activity of NOS.

*** c and nc, competitive and noncompetitive inhibition relative to BH_4 or L-arginine, respectively.

Imidazoles containing voluminous substituents displayed unexpected features. Antifungal preparations miconidazole and clotrimazole inhibited nNOS competitively with CaM [97]. Moreover, these preparations also inhibited the *in vitro* dimerization of the iNOS oxidase domain. In RAW264.7 cells 50 μM clotrimazole also inhibited the iNOS dimerization [98], unlike some substrate-like inhibitors in the presence of which the fraction of dimeric iNOS increased from 50 to 90% [16, 68]. Powerful inhibitors with similar voluminous substituents were found during the screening of compounds prepared by combination chemistry methods on the base of a pyrimidineimidazole core [99]. N-[(1,3-Benzodioxol-5-yl)methyl]-1-[2-1*H*-imidazol-1-yl)pyrimidin-4-yl]-4-(methoxycarbonyl)-piperazine-2-acetamide (BSS-1) was a highly active nNOS inhibitor not only in cell culture [100] but also in LPS-treated animals (ID_{50} = 1.2 mg/kg), as judged by decrease in nitrite content in the rat blood plasma. Note that none of these inhibitors suppressed activities of purified dimeric NOS. The search for NOS inhibitors affecting the enzyme dimerization seems promising, and this is additionally supported by finding of another imidazole-containing compound with a high inhibitory activity. 3-(2,4-Difluorophenyl)-6-{2-[4-(1*H*-imidazol-1-yl-methyl)-phenoxy]ethoxy}-2-phenylpyridine (PPA250) inhibited the NO synthesis in activated macrophages at IC_{50} = 0.08 μM and showed a therapeutic effect in experimental arthritis [101].

Nitroindazole compounds are like imidazoles in the inhibition mechanism of the inhibitor interaction with the heme. The best known compound, 7-nitroindazole (7-NI), competes with L-arginine and BH_4 in iNOS inhibition (Table 6) and with BH_4 in eNOS inhibition [90]. The competition in eNOS inhibition seems to be caused by 7-NI contact (similarly to its analog 3-Br-7-NI, $IC_{50}^{r\text{-}iNOS}$ = 0.29 μM, n/i < 1, e/i > 1 [102]) with one of the heme propionate groups, that results in suppression of the heme interaction with BH_4 [76]. Much like imidazoles, 7-NI inhibited the NADPH oxidase activity of b-nNOS [90, 103]. The strongest inhibitors of b-nNOS were 6- and 7-nitroindazoles (K_i = 40 and 2.5 μM, respectively), whereas the inhibitory activities of indazole and 5-NI were very weak (IC_{50} > 1000 μM) [104]. But according to data of one work [105], 6- and 5-NI were virtually alike in the inhibition of r-nNOS. The *in vivo* selectivity of 7-NI with respect to nNOS was due to its penetration across cell membranes and metabolism [14].

Cyclic analogs of isothiuronium. Cyclization of S-aminoalkyl-ITU resulted in the corresponding S,N-heterocycles, thiazoles, thiazolines, and thiazines [106]. 5-Methyl-2-amino-2-thiazole was a moderate inhibitor of iNOS (IC_{50} ~ 12 μM) [61]. Substitution of 2-amino-2-thiazoline in the amino group also resulted in an active eNOS inhibitor, N^5-(thiazolin-2-yl)-L-ornithine (IC_{50} ~ 1 μM) [107]. However, N-phenyl- and N-benzyl-derivatives were an order of magnitude less active [108]. Substitution of ornithine by lysine (N^{ε}-4,5-dihydrothiazol-2-yl-L-lysine) did not result in more active compounds [109]. The cyclization also resulted in 2-amino-1,3-thiazines. As in the case of N-containing heterocyclic amines, substitution in the latter position near to the heteroatom significantly increased the activity and selectivity of the resulting derivatives as compared to the initial compound. Thus, the 6-methyl-derivative [62] inhibited iNOS approximately 150-fold stronger than the parent compound 2-ADT [58]. In cell culture of RAW 264.7, 2-ADT inhibited the LPS-induced NO$^{\bullet}$ synthesis 1.5- and 4-fold stronger than 2-iminopiperidine and 2-iminohomopiperidine, although 2-ADT was less active *in vitro* than these compounds [58].

Other inhibitors. Some antithyroid drugs (thiouracils) were found to inhibit NOS. 6-Methyl-, 6-propyl-, and S-methyl-2-thiouracils displayed certain selectivity in r-nNOS inhibition [110]. The inhibition was competitive and reversible, and the effect was determined by suppression of the BH_4-dependent nNOS dimerization [111, 112]. A unique inorganic inhibitor competing with L-arginine was described in [113]. Ni^{2+} suppressed the r-nNOS activity at K_i = 30 μM, and the inhibition was partly determined by the cation effect on the binding of CaM to the enzyme.

Some bicyclic compounds representing new classes of chemical structures for designing NOS inhibitors were prepared by screening of the chemical library [114]. Values of $IC_{50}^{m\text{-}iNOS}$ for derivatives 2-benzimidazolone (FR038251), 1,3-isoquinolindione (FR038470), and 2,4-quinazolonedione (FR191863) were ~2, 9, and 2 μM, respectively. The selectivities of FR038251 and FR191863 for r-nNOS (n/i) were > 10 and to b-eNOS > 1. These compounds were also active in cell culture and in suppression of the LPS-induced nitrite production in animals' blood plasma. Active h-iNOS inhibitors (IC_{50} < 10 μM) but nonselective were prepared from benzoxazolones. The most active of these compounds, N-morpholinomethyl-6-chlorobenzoxazolone, had the following characteristics: IC_{50} = 0.8 μM, n/i > 1, e/i > 10 [115]. NOS-inhibiting activity was found in 1,2-diaminobenzimidazoles [116]. The parent compound competitively inhibited h-NOS with minimal selectivity to h-iNOS. However, the 1-methylamino-derivative was more selective to h-nNOS (IC_{50} = 6 μM, n/i < 0.1, e/i < 0.1). Ethyl- and isopropyl-derivatives were inactive.

1,2-Dihydroquinazolinamines and their spirocyclic derivatives have been recently found to have iNOS-inhibiting activity [117]. 1-(6-Cyano)-3-pyridylcarbonyl)-5′,8′-difluorospiro[piperidine-4,2′(1′H)-quinazoline]-4′-amine hydrochloride (AR-C102222) was highly effective both *in vitro* and *in vivo*: $IC_{50}^{h\text{-}iNOS}$ = 0.04 μM, n/i > 10, e/i > 1000; ID_{50} = 3 μmol/kg for the LPS-induced nitrite production in rat blood plasma. The effect of this compound in adjuvant-induced arthritis was com-

parable to the effect of the known antiinflammatory drug indomethacin.

Thus, effective NOS inhibitors capable of interacting with the enzyme catalytic center need to have: a) the guanidine, carboxamidine, or isothiuronium group with a linear or cyclic structure:

$$-NH-\underset{|}{C}=NH,$$

which provides the hydrogen bond with Glu and the coordination bond with the iron atom of the heme; b) small hydrophobic radicals as substituents in the guanidine-like group (the alkyl, phenyl, or thienyl group) which can provide additional bonds with peptide motifs proximal to the heme.

Functional substituents capable of binding to peptides located in the substrate availability channel and further from the catalytic center are significant for selectivity of the inhibitors. A chemical bridge binding the guanidine-like group and the functional residue must have certain length and mobility to ensure the optimal binding of the inhibitor to the catalytic center and outside it. The *in vivo* effectiveness of NOS inhibitors can be also favored by suppression of the superoxide synthase activity of the enzyme, regulation of the NOS dimerization, and inactivation of ROS produced by NOS (Fig. 3). It seems that in the next stage in searching for pharmaceutical agents

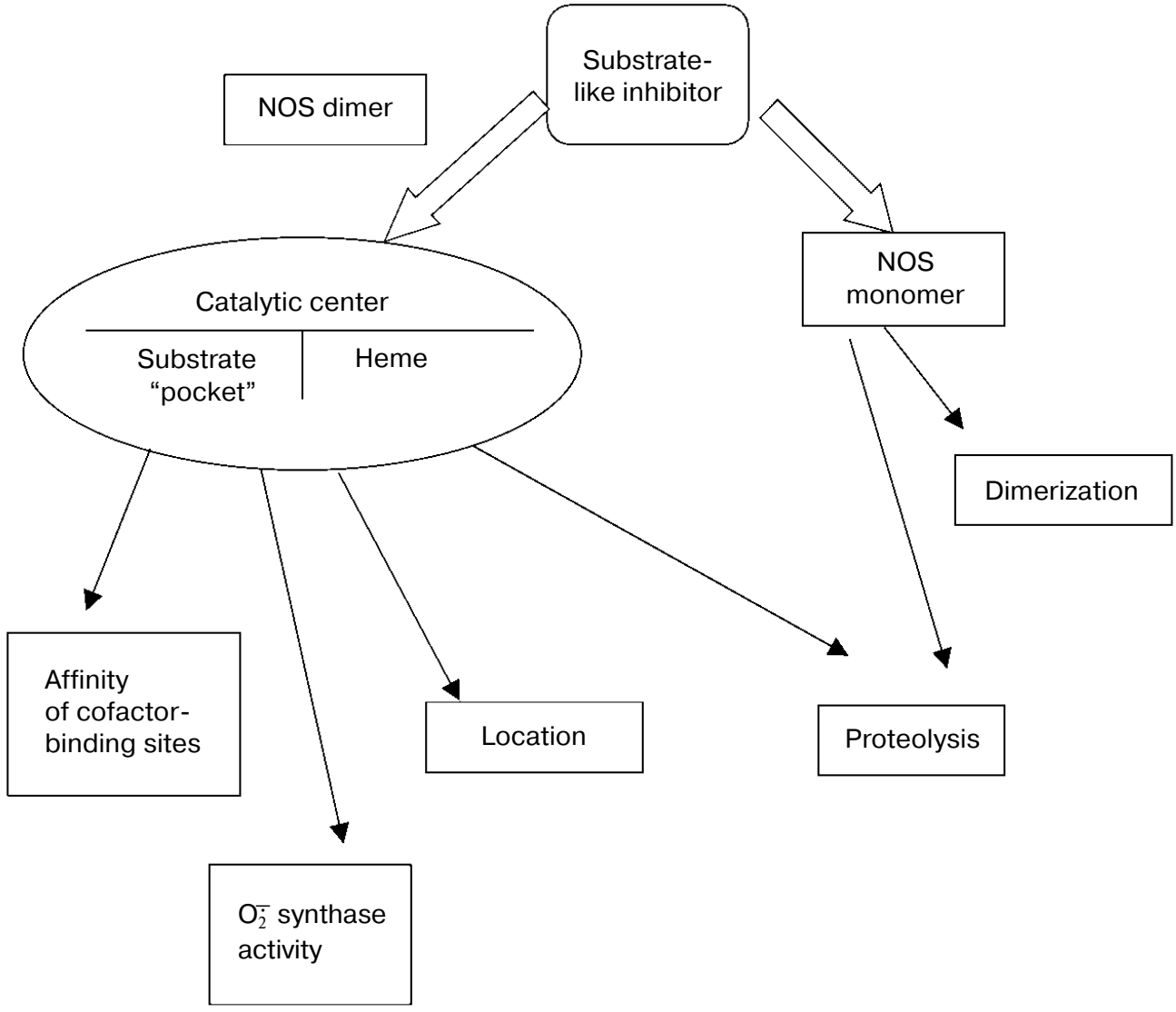


Fig. 3. Some biochemical, molecular, and subcellular targets of L-arginine-like NOS inhibitors. Interacting with the substrate-binding site, with the heme inside the NOS dimer or monomer, and with peptide motifs outside the catalytic center, these compounds can differently inhibit the NO-synthase NOS activity: by promoting the association or dissociation of polypeptides, accelerating their proteolysis, modifying their $O_2^{\cdot-}$ synthase activity, influencing the enzyme location in the cell.

modifying the enzymatic production of NO˙ compounds will be created which will combine several functions, including the specificity to NOS isoforms and antioxidant activity, as well as specific accumulation in subcellular structures (aveoles, synapses, mitochondria, etc.).

The authors are grateful to F. A. Trofimov, T. M. Roshchina, and V. Yu. Kovtun for useful discussions and remarks.

This work was supported by the Russian Foundation for Basic Research (project Nos. 01-04-4942, 02-04-50011, 03-04-48349, and 04-04-48218), the Kaluga Region Government (project No. 04-04-97238), the International Research Center (project No. 779B), and the Russian State budget.

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