Russian Chemical Reviews **74** (9) 859 – 870 (2005) © 2005 Russian Academy of Sciences and Turpion Ltd

DOI 10.1070/RC2005v074n09ABEH000923

Nitric oxide synthase inhibitors containing the carboxamidine group or its isosteres

S Ya Proskuryakov, A G Konoplyannikov, V G Skvortzov, A A Mandrugin, V M Fedoseev

Contents

I.	Introduction	859
II.	L-Arginine-dependent NO• synthesis	859
III.	Structures and activity of nitric oxide synthase inhibitors	860
IV.	Cyclic analogues of substrate-like inhibitors	865
V.	Conclusion	868

Abstract. The review summarises structures, activities and selectivity of NO-synthase (NOS) inhibitors belonging to various classes of chemical compounds. Linear, cyclic and heterocyclic structures containing guanidine, amidine and/or isothiourea fragments are considered. The structure – activity relationships for these inhibitors were analysed in relation to their action on the inducible NOS isoform. This analysis can provide the basis for the synthesis of new more efficient compounds. The bibliography includes 115 references.

I. Introduction

The fact that nitrogen(II) oxide is an endothelium-derived relaxing factor (EDRF) and the enzymatic origin of nitric oxide were both established more than 15 years ago.[1,2] Since then, it has been found that the NO• radical has unique functions in the functioning of organisms both in normal and various pathophysiological states (ischemic, inflammatory and degenerative processes, cancerogenesis).[3–6] These findings have stimulated a search for and the synthesis of new compounds capable of regulating the activity and NO• levels in animal and human organisms with the aim of using these compounds in therapy of many widespread diseases.[7,8]

Since some diseases are characterised by increased NO• production (arthritis, diabetes, sepsis, myocardial ischemia), a decrease in NO• levels is of importance in the treatment of these diseases. The available inhibitors of nitric oxide synthase (NOS) isoforms (synthase is an enzyme that catalyses the NO• synthesis) are insufficiently active and selective and cannot be localised in various tissues and cell organelles. Hence the search for new chemical NOS inhibitors is a topical problem.[9,10]

Taking into account that L-arginine is virtually the only endogenous substrate, whose guanidine group provides a nitrogen source for the NO• synthesis in enzymatic oxidation, certain analogues of this amino acid would be expected to serve as highly specific substrates for NOS enzymes. Hence, the synthesis of compounds containing groups isosteric to the guanidine group of L-arginine attracts the most attention. In the present review, we analyse the data on the relationships between the chemical structures and biochemical activities of inhibitors that bind specifically to the catalytic site of the enzyme, where L-arginine is accepted and oxidised. The data published in 1997 – 2003 are summarised. The properties of inhibitors are considered in relation to the inducible NOS (iNOS) isoform, which not only plays a key role in the regulation of nonspecific immunity[4] but also can suppress defence mechanisms in pathogenesis of certain diseases by synthesising excess NO•.[8,11,12]

S Ya Proskuryakov, A G Konoplyannikov, V G Skvortzov Medical Radiological Research Centre, Russian Academy of Medical Sciences, ul. Koroleva 4, 249036 Obninsk, Russian Federation. Fax (7-095) 956 14 40, tel. (7-08439) 7 48 63, e-mail: pros@mrrc.obninsk.ru, noo@mrrc.obninsk.ru (S Ya Proskuryakov), tel. (7-08439) 7 20 54, e-mail: konopl@mrrc.obninsk.ru (A G Konoplyannikov), tel. (7-08439) 7 40 65, e-mail: skvortsov@mrrc.obninsk.ru (V G Skvortzov)
A A Mandrugin, V M Fedoseev Department of Chemistry, M V Lomonosov Moscow State University, Leninskie Gory, 119992 Moscow, Russian Federation. Fax (7-095) 939 31 87, tel. (7-095) 939 38 45, e-mail: mandrug@radio.chem.msu.ru (A A Mandrugin), tel. (7-095) 939 31 87, e-mail: fedoseev@radio.chem.msu.ru (V M Fedoseev)

Received 15 March 2004
Uspekhi Khimii **74** (9) 939 – 952 (2005); translated by T N Safonova

II. L-Arginine-dependent NO• synthesis

In living organisms, NO• is produced primarily by enzymatic oxidation of L-arginine,[13,14] although there are other sources of this radical.[15] The following three forms of the enzymes synthesising NO• are distinguished: two constitutive forms and one inducible form, which is expressed by anti-inflammatory cytokines and bacterial products. The isoforms are encoded by distinct genes and are named for their predominant localisation: neuronal NOS (NOS-1, nNOS), inducible NOS (NOS-2, iNOS) and endothelial NOS (NOS-3, eNOS).

1. Substrates and cofactors

The following three substrates are involved in the NO• synthesis performed by the homodimeric enzyme: L-arginine (R = NH), oxygen and nicotinamide adenine dinucleotide phosphate (NADPH) as the electron source. Two-step oxidation of the amino acid occurs through the intermediate formation of N^{ω}-hydroxy-L-arginine (R = NOH) followed by the formation of L-citrulline (R = O) and NO• in equimolar amounts.[13]

$$R{=}NH + NADPH + H^+ + O_2 \longrightarrow$$
$$\longrightarrow R{=}NOH + NADP^+ + H_2O,$$
$$R{=}NOH + 0.5\,NADPH + 0.5\,H^+ + O_2 \longrightarrow$$
$$\longrightarrow R{=}O + 0.5\,NADP^+ + H_2O + NO^{\bullet}.$$

The activity of NOS is mediated by several cofactors and prosthetic groups. Some of them, for example, FAD, FMN, a haem with pentacoordinate Fe^{2+}, the Ca^{2+}-binding protein (calmoduline, CaM) and Zn^{2+}, are involved in each subunit. There is certain evidence that cofactors other than (6*R*)-5,6,7,8-tetrahydro-L-biopterin (BH_4) can be absent in the dimer.[14] Flavins involved in the reductase domain transfer electrons from NADPH to the oxidase domain containing the haem (oxidation site), BH_4- and L-arginine-binding regions and a zinc ion.[16]

2. Dimerisation and proteolysis of NOS

Nitric oxide synthase oxidises L-arginine and synthesises NO$^{\bullet}$ only in the form of the homodimeric complex, L-arginine, BH_4 and the haem being altogether involved in the formation of the active form of the enzyme. Hence, the replacement of the substrate with the inhibitor not only inhibits the NO$^{\bullet}$ synthesis but also often hinders the formation of the active tertiary structure of NOS.[14, 16, 17] In intact cells, ~50% of NOS polypeptides exist as dimers.[18] The involvement of the haem in an NOS polypeptide is apparently the first necessary step of dimerisation for all isoforms.[19] However, the role of BH_4 and L-arginine is more differentiated. In particular, the substrate is more efficient in association of nNOS subunits than BH_4 . However, this dependence for iNOS is inverse. On the whole, in the presence of the above-mentioned cofactors and the substrate, resistance of the dimer to dissociation and proteolysis decreases in the following series of NOS isoforms: eNOS $\gg$ nNOS > iNOS.[20] However, noteworthy is the unexpected fact of the possible binding of L-arginine to the BH_4-binding site of eNOS.[21]

3. Structure of the oxidase domain of NOS

The oxidase domain of NOS contains four important structure fragments of the enzyme: the oxidation site containing the haem with pentacoordinate iron, the L-arginine-binding region, the BH_4-accepting pocket, and the Zn^{+2} thiolate complex. Two conservative amino acids play the central role in L-arginine binding. A proton of one of the amino groups (from the guanidine structure) of L-arginine is accepted by the carbonyl group of tryptophan in the haem proximal pocket. In the distal pocket, the carbonyl group of glutamic acid binds the second proton of the same amino group and the proton attached to the N(5) atom.[21] L-Arginine is apparently involved in the accepting pocket of the oxidase domain in the extended *E* conformation.[22] Figure 1 shows the arrangement of one of NOS inhibitors, *viz*., N^{ω}-n-propyl-L-arginine (PLA), in the guanidine-binding pocket of NOS[23, 24] and indicates two main amino acids of the accepting pocket, Glu and Trp. Substrate-like inhibitors can interact with the oxidation pocket containing the haem with the penta- or hexacoordinate iron ion, resulting in either the displacement of the sixth ligand (without the bond formation) or the formation of the sixth coordination bond with the haem iron ion (for example, L-thiocitrulline). The carbonyl groups of Trp and Glu are involved in PLA binding analogously to L-arginine binding. One of the axial bonds of penta- or hexacoordinate Fe^{2+} is formed by the cysteine residue from the NOS polypeptide, whereas another bond can be formed with an inhibitor, which substantially affects its efficiency.

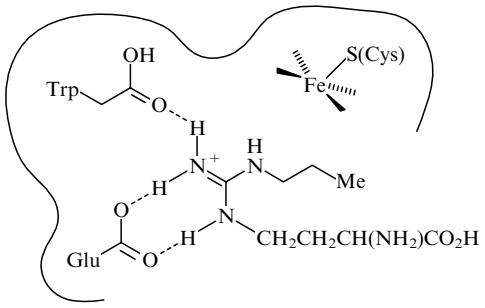


Figure 1. Scheme of assumed interactions of N^{ω}-*n*-propyl-L-arginine (a substrate-like NOS inhibitor) with the catalytic site of NO synthase, where binding and oxidation of the substrate (L-arginine) occurs.

Analysis of the relationships between the sizes of the substituents in L-arginine-like inhibitors and stability of inhibitor–NOS complexes (inhibitor analysis) and investigation of the spectroscopic characteristics of nNOS, iNOS and eNOS demonstrated that the size of the substrate pocket of the isoforms increases in the following series: nNOS > iNOS > eNOS.[25, 26] In addition, the discriminative topological features of the distal and proximal pockets accepting guanidine amino groups were revealed. The binding region for the amino group (which serves as the nitrogen source for NO$^{\bullet}$) can interact with fragments substantially bulkier than the NH_2 or =NOH groups. In contrast, the binding site of the distal nitrogen cannot accommodate such groups. These topological features of the substrate-binding pocket are also retained for the isoforms. Thus, iNOS reacts only weakly with *N*-alkyl groups larger than the ethyl group in the vicinity of the haem and cannot accept the SR group in the distal guanidine-binding pocket. At the same time, inhibitors containing larger SAlk or NAlk groups are more strongly bound to the catalytic site of nNOS and, consequently, more strongly and selectively inhibit nNOS than iNOS.[24]

It should be noted that, in spite of the structural features of the catalytic sites of the NOS isoforms, peptide regions located in the haem access channel play apparently a more essential role in selective inhibitor binding to the enzyme. It is these regions that accept BH_4 and determine dimerisation of monomers, their covalent modification and localisation of the protein in cells. These structural features, which remain unstudied by X-ray diffraction analysis, hold promise for the design of highly selective and active NOS inhibitors.

III. Structures and activity of nitric oxide synthase inhibitors

Since the discovery of the enzymatic origin of NO$^{\bullet}$, hundreds of compounds have been studied as NOS inhibitors using the isolated enzyme with different degrees of purity. However, these necessary and useful data were obtained in the absence of commonly accepted standards for the analysis of the species origin of the enzymes, procedures for their isolation, reaction conditions, *etc*. In addition, estimates for the inhibiting activity of compounds on isolated enzymes ruled out a number of important mechanisms, which can determine the efficiency of inhibitor at the cell level. Among them are, for example, modifications of the enzyme stability to proteolysis, the metabolism rate of inhibitors, the cell membrane permeability, *etc*.

The activity of nitric oxide synthase inhibitors is generally characterised by the stability of an inhibitor–enzyme complex using the K_i (see †) and IC_{50} parameters ‡ determined from the kinetic data. The compounds are classified into four groups according to the activity. For these groups, K_i (IC_{50}) vary in the ranges >100, 10–100, <10 and <1 μM, respectively. For example, the Michaelis–Menten constant of NOS for arginine K_M^{Arg} = 1–10 μM,[27] whereas the corresponding constant for L-homoarginine is approximately 10 times larger. The data on selective inhibition of a particular NOS isoform are represented as the K_i^{eNOS}/K_i^{iNOS} (e/i) and K_i^{nNOS}/K_i^{iNOS} (n/i) ratios (or as the corresponding IC_{50} ratios). The inhibitors are also divided into four groups according to the numerical values of the 'selectivity'

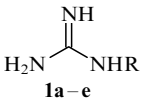
† The inhibition constants K_i (assuming that the reaction is competitive and stationary) are calculated by the equation $1/v = 1/V_{max} + (K_M^{Arg}/V_{max}[Arg]) \times (1 + [I]/K_i)$, where $[I]$ and $[Arg]$ are the concentrations of the inhibitor and arginine, respectively; K_M^{Arg} is the Michaelis–Menten constant.

‡ IC_{50} is the inhibitor concentration at which the NOS activity is suppressed by 50% at a given arginine concentration.

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. Except for the abbreviated names of NOS inhibitors proposed by the International Union of Pharmacology Nomenclature,[28] other abbreviated names and notations correspond to those reported in original works.

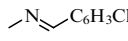
1. Guanidine and L-arginine derivatives

Aminoguanidine is one of the structurally simple and most well-known NOS inhibitors. This compound inhibits all NOS isoforms and exhibits certain selectivity for iNOS. The *in vivo* action of this inhibitor is difficult to interpret because of the presence of alternative biochemical targets (see comments to the paper [13]). Aminoguanidine (**1a**), like other guanidine derivatives, irreversibly inactivates all NOS isoforms (Table 1).

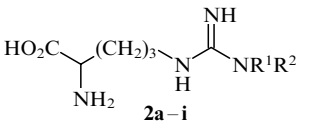


1a–e

R = NH_2 (**a**), Me (**b**), Et (**c**), Ph (**d**),

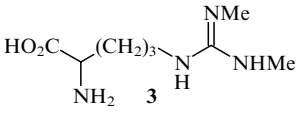


(**e**).

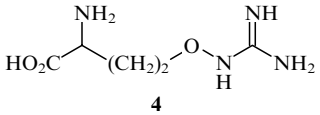


2a–i

R^1 = H: R^2 = NO_2 (**a**), NH_2 (**b**), Me (**c**), Pr^n (**d**), Bu^n (**e**), cyclo-C_3H_5 (**f**), All (**g**), $CH_2C{\equiv}CH$ (**h**); $R^1 = R^2$ = Me (**i**).



3



4

The replacement of the amino group in compound **1a** with the methyl group noticeably decreases the inhibiting activity of *N*-methylguanidine (**1b**) ($IC_{50}^{iNOS} \approx 100$ μM).[31] Of 1-substituted guanidines, *N*-ethylguanidine (**1c**) proved to be the strongest iNOS inhibitor. Compound **1c**, unlike compound **1a**, appeared to be more selective for this isoform.

The known pharmaceutical vasorelaxant agent, an α_2-adrenoreceptor agonist, *viz*., 1-(2,6-dichlorobenzylideneamino)guanidine (**1e**), was found to inhibit NOS.[35] In the initial period, this agent reversibly inhibits the enzyme in HEK 293 cell cytosol ($K_i^{nNOS} = 1$ μM), but later on, irreversible inactivation of nNOS occurs. In the presence of compound **1e**, the lifetime of nNOS in the cell culture decreased from 20 to 10 h.[36]

L-Arginine derivatives (**2a–i**) containing the substituted guanidine group were the first, which proved to be NOS inhibitors. It should be emphasised that their D isomers have no effect on the enzymatic activity of NOS.[24] According to the iNOS-inhibiting activity of N^{ω}-nitro-L-arginine (**2a**), the latter can be assigned to strong inhibitors ($K_i < 10$ μM, see Table 1). The activity of nNOS and eNOS is suppressed to a greater degree compared to iNOS due to the difference in the mechanisms of binding to the enzyme isoforms. Binding to iNOS, as well as the replacement with L-arginine, occur rapidly. Binding of the inhibitor to constitutive NOS occurs slowly, and the inhibitor is even more slowly separated from the enzymes in the presence of a substrate.[37,38] The amino derivative of arginine **2b** also inhibits NOS both *in vitro* and *in vivo* through either irreversible removal of the haem from the polypeptide or covalent binding of the haem to the polypeptide.[39]

Active compounds were also found among monoalkylarginines. N^{ω}-Methyl-L-arginine (**2c**), like nitro derivative **2a**, serves as an efficient inhibitor of all NOS isoforms without pronounced selectivity. The mechanism of action of compound **2c** as an inhibitor involves inactivation of the enzyme, compound **2** being hydroxylated as a pseudosubstrate with NOS analogously to L-arginine. However, products formed in the next step ($MeNO^+$, CH_2O, *etc*.), covalently modify the enzyme, which is apparently accompanied by removal of the haem.[40–42] It should be noted that N^{ω}-methyl-L-arginine (**2c**) and N^{ω},N^{ω}-dimethyl-L-arginine (**2i**) (asymmetric dimethylarginine) are endogenous NOS inhibitors formed in living organisms. Derivatives **2c**,**i** have approximately equal activity ($K_i^{nNOS} \approx 0.5$ and 0.7 μM, respectively),[43] which is attributed to the fact that one of the amino groups of the guanidine fragment of L-arginine is not bound to the substrate-binding region (see Fig. 1). By contrast, symmetric N^{ω},N^{ω}-dimethyl-L-arginine (**3**) is a very weak inhibitor.

In a series of L-arginine derivatives containing aliphatic substituents in the N^{ω} position, the iNOS-inhibiting activity of the compounds decreases substantially as the substituent size increases [> 4000 μM for N^{ω}-n-butyl-L-arginine (**2e**)], but the nNOS-inhibiting activity increases.[24] For example, N^{ω}-n-propyl-arginine (**2d**), being a weak iNOS inhibitor ($IC_{50}^{iNOS} \approx 950$ μM), exhibited high selectivity for nNOS (n/i < 0.001) [31] and moderate selectivity for eNOS (e/i < 0.1). N^{ω}-Cyclopropyl-L-arginine (**2f**) appeared to be a strong m-iNOS inhibitor ($K_i^{iNOS} \approx 8$ μM).[42]

The appearance of a multiple bond in the alkyl substituent of arginine [in N^{ω}-allyl-L-arginine (**2g**) and N^{ω}-propargyl-L-arginine (**2h**)] is accompanied by a decrease in their nNOS-inhibiting activity and an increase in the iNOS- and eNOS-inhibiting activity. Apparently, this is associated with inactivation of iNOS under the action of inhibitors with a multiple bond.[44]

The amino acid of plant origin, L-canavanine (**4**), in which the δ-methylene group of L-arginine is replaced with the oxygen atom, weakly and non-selectively inhibits the $NO^{\bullet}$ synthesis ($IC_{50}^{nNOS} \approx 130$ μM). This is apparently attributed to the fact that a hydrogen bond involving the hydrogen atom at the N(5) atom cannot exist, because the labile hydrogen is absent in the favourable tautomeric form of L-canavanine.[24] However, physiological experiments provided evidence for specific activity of this amino acid against iNOS. In particular, the hypertensive effect of the inhibitor on animals treated with lipopolysaccharide (with the iNOS inducer) was substantially higher than that on the intact

Table 1. Inhibiting activity of guanidine and L-arginine derivatives.

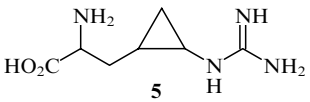
Compound	K_i^{iNOS} /μM (see [a])	Selectivity (see [b])		Ref.
		n/i	e/i	
1a	30 [c]	6	10	14
	30 [d]	12	9	28
	5 [e]	> 1	> 1	31
1c	1 [e]	> 10	> 10	31
2a	3 [c]	0.09	< 0.1	14
	2 [c,d]	0.02	0.05	28
	13 [e]	0.2	0.09	31
	0.7 [f]	0.02	0.04	32
2c	7	0.7	0.5	14
	5 [d]	0.2	0.2	24
	1.3 [d]	1	0.3	29
	1 [f]	1	0.5	33
2e	100 [d]	0.004	0.05	24
	25 [d]	0.02	0.08	34
2h	0.6 [g]	> 1	> 1	34

Note. Hereinafter, the averaged data are given, which can be used for comparative analysis only within the scope of the present review.

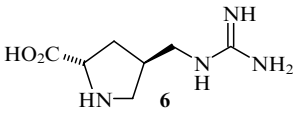
[a] In the cases noted, the constants K_i were calculated from the data reported in the corresponding studies according to the equation $IC_{50} = K_i(1 + [Arg])/K_M^{Arg}$. [b] The selectivity (predominant inhibition of one NOS isoform compared to another isoform) is expressed as the ratio of the values characterising the activity of the inhibitor, IC_{50} or K_i (n/i = $IC_{50}^{nNOS}/IC_{50}^{iNOS}$, e/i = $IC_{50}^{eNOS}/IC_{50}^{iNOS}$). [c] h-NOS, IK_{50}; [Arg] = 30 μM (the species origin of NOS: m, r, b, h are mouse, rat, bovine and human, respectively). [d] K_i was calculated from the data published in the study.[24,29,30] [e] m-i NOS, r-nNOS, b-eNOS. [f] h-NOS. [g] m-i NOS, b-nNOS, b-eNOS, IK_{50}; [Arg] = 120 μM.

animals, in which the vascular tone is regulated by $NO^{\bullet}$ produced by eNOS.[45]

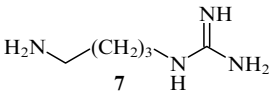
The NO-inhibiting properties of derivatives **5**–**7**, which are structurally similar to L-arginine, contain a substituent in the amino acid moiety and are characterised by the same structure of the guanidine group, were studied.[46]



5



6



7

It appeared that, of the conformational isomers of (±)-*trans*-3(2-guanidino)cyclopropylalanine (**5**), *syn*,*trans*-guanidinocyclopropylalanine is a poor substrate, whereas the *anti* isomer is a weak NOS inhibitor. Amino acid **6**, in which the ornithine fragment of L-arginine is replaced with L-proline, is also a weak NOS inhibitor.[47] The absence of the amino acid moiety does not hinder the formation of a complex of the inhibitor with the guanidine-binding region, as evidenced by abundant data. However, the amino acid fragment limits the number of isomers (generally, of the L form), which can bind to the catalytic site of the enzyme.

Studies of 1-amino-4-guanidinobutane [agmatine (**7**)], which is one of the products of L-arginine metabolism, revealed new NOS-inhibiting properties. Agmatine (**7**) enhances oxidation of NADPH and facilitates the formation of H_2O_2 by the isomer of nNOS, the latter being inactivated. In the reversible initial inhibition step, the affinity of agmatine (**7**) for nNOS was characterised by $K_i^{nNOS} \approx 30$ μM.[48]

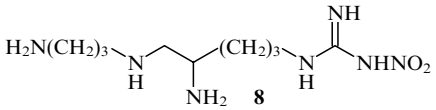
Considering the satisfactory inhibiting activity of compound **2a**, N^{ω}-nitro-L(D)-arginine derivatives as dipeptides containing D and L isomers of phenylalanine were synthesised and characterised. The inhibition constants ($K_i^{iNOS} > 10$ μM) show that these compounds can be considered as weak inhibitors. Of these compounds, N^{ω}-nitro-L-arginyl-L-phenylalanine benzyl ester, which is conditionally selective for iNOS, is most active against iNOS ($K_i^{iNOS} = 18$ μM, n/i and e/i > 1).

At the same time, compounds with unique selectivity for nNOS and eNOS were found among inefficient iNOS inhibitors ($K_i^{iNOS} > 1000$ μM) belonging to this series. In particular, D-phenylalanyl-N^{ω}-nitro-D-arginine methyl ester selectively inhibits nNOS and eNOS compared to iNOS ($K_i^{iNOS} = 3600$ μM, n/i < 0.001, e/i < 0.01). It should be noted that dipeptides containing the nitro-D-arginine (D-NA) and D/L-phenylalanine (D/L-Phe) fragments proved to be iNOS inhibitors uncompetitive with L-arginine, but they are competitive nNOS and eNOS inhibitors.[49, 50] These data suggest that there are specific binding sites in iNOS outside the guanidine-binding pocket, which can accept amino acid fragments with an unnatural geometry.

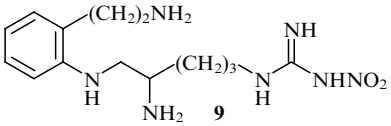
More active and selective inhibitors were prepared by the replacement of the carboxy group with the amide group in dipeptides. Amides of N^{ω}-nitro-L-arginyl-D-asparagine ($K_i^{iNOS} = 9$ μM, n/i < 0.1, e/i > 10) and N^{ω}-nitro-L-arginyl-D-2,4-diaminobutanoic acid ($K_i^{iNOS} = 25$ μM, n/i < 0.01, e/i > 1) exhibited exclusive selectivity for nNOS compared to eNOS. One of the least active iNOS inhibitors, *viz*., L-lysyl-N^{ω}-nitro-D-arginine amide, exhibited selectivity for all three isoforms ($K_i^{iNOS} = 4700$ μM, n/i < 0.001, e/i > 0.1).

Reduction of the amide group to the amine group in this class of stereoselective compounds afforded peptidomimetic agent **8**, which has moderate iNOS-inhibiting activity ($K_i^{iNOS} > 10$ μM) and exhibits even higher selectivity for nNOS (n/i < 0.01 and n/e < 0.001).[51] With the aim of increasing the lipophilicity of such compounds, their analogues were synthesised. One of them (compound **9**) also has exclusive activity and selectivity for nNOS ($K_i^{iNOS} = 3.5$ μM, n/i ≈ 0.01 and n/e < 0.001).[52]

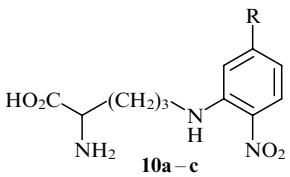
Yet another class of NOS inhibitors, which contain no carboxamide groups and are structurally similar to arginine **2a**, was found among compounds **10a**–**c**. 5-Methylphenyl-2-nitro-*N*(5)-L-ornithine (**10b**) appeared to be the most active of these compounds and exhibited moderate selectivity for nNOS ($K_i^{nNOS} = 1.4$ μM, n/i < 1, e/i < 1). Stereoselectivity of this compound, unlike that of arginine derivatives, was not pronounced. The stereoisomer of D-ornithine **10c** inhibited nNOS 30 times weaker compared to the L form of **10b**.[53]



8



9



10a–**c**

R = H (**a**), Me (L-form) (**b**); Me (D-form) (**c**).

Therefore, efficient NOS inhibitors were found among compounds containing the high-basicity guanidine group. However, the degree of binding to the enzymes is substantially affected by another fragment of the inhibitor molecule, which is responsible for its selectivity for NOS isoforms.

2. Inhibitors containing the carboxamidine group

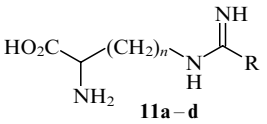
The theoretical model[24] of the L-arginine-binding pocket of NOS suggests that the oxidised amino group of the guanidine fragment is not involved in complexation of L-arginine (or a substrate-like inhibitor) with the corresponding amino acids of the catalytic site. Hence, modifications or the complete replacement of the amino group oxidised to $NO^{\bullet}$ should not influence substantially the interaction of a substrate-like inhibitor with the enzyme (see Fig. 1).

In a series of N^{ω}-(1-iminoalkyl)-L-ornithines **11a**,**b** and L-lysines (**11c**,**d**), *N*(5)-(1-iminoethyl)-L-ornithine (**11a**) is the most active iNOS inhibitor, which exhibits low selectivity for nNOS and eNOS (Table 2). An increase in the length of the alkyl substituent leads to a decrease in the activity of inhibitors of all NOS isoforms, unlike N^{ω}-alkyl-L-arginines **2**, whose affinity for nNOS increases.[24]

Table 2. Inhibiting activity of amino acid derivatives containing the amidine fragment.

Compound	K_i^{iNOS} /μM	Selectivity		Ref.
		n/i	e/i	
11a	5 [a]	< 1	< 1	24
11b	60	0.002	< 1	54
11c	2 [b]	> 0.1	> 10	50
	1 [b]	10	16	55
12a	2 [b]	500	≈ 10	56
12b	8 [b, c]	80	100	57
12c	1.4 [b]	300	100	57
12d	1 [a]	60	130	58

[a] IC_{50}. [b] K_i was calculated from the data published in the study [31].
[c] h-NOS [Arg] = 30 μM.



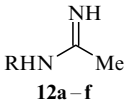
11a–d

n = 3: R = Me (**a**), All (**b**);
n = 4: R = Et (**c**), All (**d**).

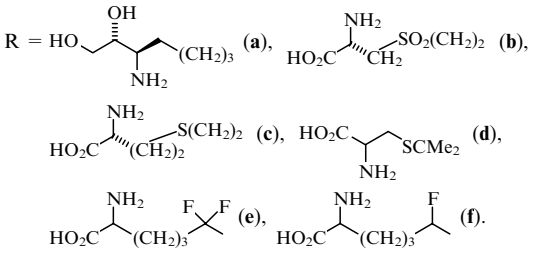
The influence of the allyl substituent on the behaviour of the L-ornithine molecule was demonstrated using *N*(5)-(1-iminobut-3-enyl)-L-ornithine (**11b**) as an example. The latter also exhibited high selectivity for nNOS. Apparently (as in the above-described cases), this is associated with an inactivation mechanism of the reaction of compound **11b** with the enzyme.

The replacement of ornithine with lysine gives rise to *N*(6)-(1-iminoethyl)-L-lysine (**11c**) having the highest iNOS-inhibiting activity. Unlike L-ornithine derivatives, the lysine derivative exhibits moderate selectivity for iNOS (see Table 2). The replacement of the ethyl substituent with the allyl group in *N*(6)-(1-iminobut-3-enyl)-L-lysine has virtually no effect on the activity and selectivity. Therefore, the aliphatic substituent at the carboxamidine group has the same effect on the iNOS-inhibiting activity of compounds **11a–d** as the aliphatic substituent at the guanidine group. However, the selectivity of inhibitors belonging to the amino-acid class depends substantially on the modification of such substituents (the insertion of an unsaturated bond) and the nature of the amino acid residue (lysine instead of ornithine).

An original series of stereospecific NOS inhibitors **12a–f** analogous to compounds **11c** was designed (see Table 2).



12a–f



(**a**), (**b**), (**c**), (**d**), (**e**), (**f**).

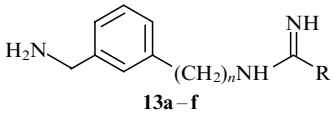
The influence of the carboxy group on the efficiency of inhibition was studied for compound **11c** and *N*-[(5*S*)-amino-(6*R*),7-dihydroxyheptyl]acetamidine (**12a**), which, although being less active against iNOS, appeared to be highly selective for this isoform (compared to eNOS).

In a series of derivatives in which the carbon atom is replaced with the S atom and the SO_2 group (**12b**,**c**), the iNOS-inhibiting activity of the L isomers is higher (IC_{50} = 0.7–1.6 μM) than that of the corresponding D isomers. Three compounds of this series, *viz*., *S*-{2-[(1-iminoethyl)amino]ethyl}-4,4-dioxo-L-cysteine (**12b**), *S*-{2-[(1-iminoethyl)amino]ethyl}-L-homocysteine (**12c**) and *S*-{2-[(1-iminoethyl)amino]isopropyl}-L-cysteine (**12d**), exhibited high selectivity for iNOS.

Fluorination of lysine at position 4 afforded the following iNOS inhibitors: *N*(5)-(1-iminoethyl)-4,4-difluoro-L-lysine (**12e**) and *N*(5)-(1-iminoethyl)-4-fluoro-L-lysine (**12f**).[59]

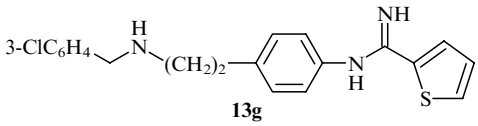
The presence of an amino acid residue is not necessary for inhibitors containing the carboxamidine group,[60] as well as for compounds containing the guanidine group.

Of non-amino acid inhibitors containing the amidine group, *N*-[3-(aminomethyl)benzyl]acetamidine (**13a**) proved to be iNOS-selective. Apparently, the activity and specificity are governed by the two-step chemical reaction of the inhibitor with the inducible enzyme. Unlike the fast and reversible reaction of arylamidine **13a** with constitutive NOS, the second step of the reaction of this inhibitor with iNOS occurs slowly and virtually irreversibly[61] (Table 3).



13a–f

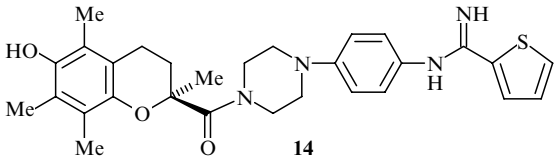
Compound **13**	R	n	Compound **13**	R	n
a	Me	1	**d**	CH_2F	0
b	Me	0	**e**	2-thienyl	0
c	$MeSCH_2$	0	**f**	2-furyl	0



13g

Of other analogues of structure **13a**, nNOS-selective inhibitors, in particular, *N*-[3-(aminomethyl)phenyl]acetamidine (**13b**), were found. These inhibitors differ from compound **13a** by one methylene group. In this class of compounds, the inclusion of the thiomethyl group into the acetamidine fragment in *N*-[3-(aminomethyl)phenyl]methylthioacetamidine (**13c**) gives rise to the most active inhibitor of both iNOS and nNOS. Compound **13d** containing the fluoromethyl substituent in the acetamidine group is also a highly efficient nNOS inhibitor. Analogously, the replacement of the acetamidine group with the hetarylamidine group (**13e**,**f**) increases the nNOS-inhibiting activity of the resulting compounds (K_i^{nNOS} = 6 and 9 nM, respectively). Of other thiophene-2-carboxamidines, *N*-{4-[2-([(3-chlorophenyl)methyl]-amino)ethyl]phenyl}thiophene-2-carboxamidine **13g** is a highly efficient iNOS inhibitor (see Table 3), in which the thiophene fragment proved itself as a promising pharmacophore.[15]

The presence of the thiophenecarboxamidine group and the active site of the vitamin E in one molecule made it possible to prepare highly selective nNOS inhibitor **14** ($K_i^{r-nNOS} \approx 0.9$, $K_i^{b-eNOS} \approx 110$, $K_i^{m-iNOS} > 300$ μM) and the antioxidant BN80933 exhibiting the anti-ischemic activity.[31,62]



14

Among syntheses of compounds biologically active *in vivo*, noteworthy is the synthesis of drugs, *i.e*., substances with useful pharmacological properties (necessary lipophily, solubility, *etc*.), which undergo metabolism with elimination of an NOS inhibitor in living organisms. For example, *N*(6)-(1-iminoethyl)-L-lysine *N*-tetrazol-5-ylamide **11c** showed efficiency comparable with that of compound **11a** in a model of endotoxin shock and arthritis.[63]

Table 3. Inhibiting activity of non-amino acid compounds containing the amidine group.

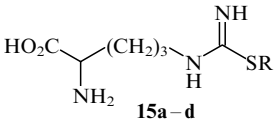
Compound **13**	K_i^{iNOS} /μM	Selectivity		Ref.
		n/i	e/i	
a	0.14	> 10	≈ 500	29
	0.2 [a,b]	> 10	> 1000	14
b	2 [c]	0.02	> 1	29
c	0.02 [c]	< 1	> 10	29
g	5 [a,b]	0.004	< 1	12
	0.3 [a]	< 1	> 1	14

[a] IC_{50}. [b] h-NOS, [Arg] = 30 μM. [c] h-NOS. [d] m-i NOS, r-nNOS, h-eNOS.

The above data demonstrate that a decrease in the basicity of the guanidine-like group (which determines binding of the inhibitor to the enzyme) as a result of the complete replacement of one amino group does not decrease the inhibiting activity. Moreover, more active compounds **13a**,**c**,**g** were prepared by modifications of the carboxamidine residue with aryl and hetaryl fragments.

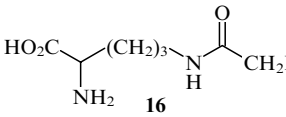
3. L-Thiocitrulline and its isothiocarbamide analogues

L-Citrulline and its analogues **15**–**17** belong to a vast class of compounds in which one nitrogen atom of the guanidine group is replaced by the oxygen or sulfur atom. L-Citrulline, which is one of the products of enzymatic oxidation of L-arginine, is a very weak NOS inhibitor.[24] However, L-thiocitrulline (**15a**) proved to be a strong inhibitor, nNOS being inhibited ~50 times stronger than iNOS (Table 4).



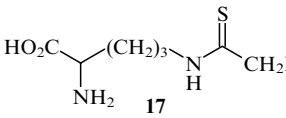
15a–**d**

R = H (**a**), Me (L form) (**b**), Me (D form) (**c**), Ph (L form) (**d**).



16

R = Me, Et, Pr^n, Bu^n.



17

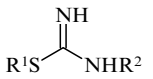
R = Me, Et, Pr^n, Bu^n.

One of the mechanisms determining the strength of affinity of compound **15a** to the neuronal isoform of the enzyme involves binding between the Fe atom of the haem and the thiol group of the inhibitor. This fact was observed spectrophotometrically (a type-II differential spectrum of the haem recorded upon binding of compounds as the sixth ligand of the hexacoordinate Fe atom).

The introduction of the methyl substituent into the thiol group of L-thiocitrulline **15a** giving rise to *S*-methyl-L-thiocitrulline (**15b**) substantially strengthens binding to the enzyme and simultaneously decreases selectivity. A further increase in the size of the aliphatic substituent leads to a decrease in the inhibiting activity by a factor of *ca.* 50. Unlike binding of L-thiocitrulline **15a**, binding of its aliphatic derivatives to the enzyme was characterised by a type I spectral shift (*i.e.*, by the differential spectrum analogous to the spectrum of the haem upon the formation of the L-arginine–enzyme complex). This reflects the displacement of the sixth ligand from the hexacoordinate haem iron without bonding with the latter.[64] By contrast to N^{ω}-alkyl-L-arginines, stereoselectivity was not absolutely necessary for binding this group of inhibitors to the enzyme. *S*-Methyl-D-thiocitrulline (**15c**) inhibits, although weakly, iNOS and nNOS ($IC_{50} \approx 470$ and 160 μM, respectively). *S*-Methyl-L-thiocitrullinyl-L-phenylalanine appeared to be a strong and selective iNOS inhibitor ($IC_{50} = 0.65$ μM, n/i ≈ 60).[67]

The replacement of the second amino group in L-citrulline or L-thiocitrulline with the alkyl group leads to a virtually complete loss (a decrease by two orders of magnitude) of the inhibiting activity of these compounds. For *N*(5)-acyl-L-ornithines **16** and *N*(5)-thioacyl-L-ornithines **17**, $IC_{50} \geqslant 1000$ μM. This is indicative of a weak affinity of the sulfur and oxygen heteroatoms for the catalytic site, if the alkyl group is at position 6 of the molecule. Therefore, for a strong complex with the enzyme to be formed, it is necessary that the inhibitor should contain the second NH group that forms the carboxamidine fragment.[24]

In compounds containing the isothiocarbamide group, as in guanidines and carboxamidines, the amino acid moiety is not a necessary component for the design of efficient inhibitors. Exceptions are thiocarbamide, in which the nitrogen atom bears the terminal L-thiocitrulline residue ($K_i > 2500$ μM), and its *N*-methyl derivatives. Their inhibiting activity is substantially lower (by > 500 times) than that of the corresponding *S*-alkyl derivatives. Ethyl- (**18b**) and isopropylisothiocarbamides (**18c**) are weakly selective for this isoform and proved to be the most active iNOS inhibitors (see Table 4).



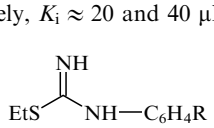
18a–**e**

Compound **18**	R^1	R^2
a	$(CH_2)_2NH_2$	H
b	Et	H
c	Pr^i	H
d	$(CH_2)_2C_6H_4OMe$-3	H
e	H	Ph

All *S*-alkylisothiocarbamides are competitive reversible inhibitors; they are simultaneously substrates for the haem.[34, 65, 66] *S*-Ethylisothiocarbamide promotes dimerisation of iNOS to a greater degree even compared to L-arginine.[68] *S*-(2-Aminoethyl)-isothiocarbamide (**18a**), like aminoguanidine **1a**, inhibits expression of iNOS in mouse macrophages and rat lungs.[69] In aqueous solutions (pH 7), *S*-aminoalkylisothiocarbamides are rearranged into the corresponding mercaptoalkylguanidines,[70] which are also iNOS inhibitors.[71]

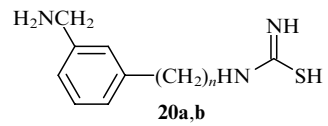
Among *S*-phenylalkyl derivatives of isothiocarbamides, only compound **18d** exhibited selectivity, which was manifested in weaker inhibition (by a factor of 20) of b-eNOS compared to m-iNOS.[72]

N-Phenyl derivatives more actively inhibit nNOS. At the same time, *N*-phenylisothiocarbamide (**18e**) exhibited weak selectivity ($K_i^{iNOS} \approx 0.9$ μM, n/i ≈ 0.1 and e/i ≈ 0.5). It should be noted that *S*-ethyl-*N*-phenylisothiocarbamide derivatives are less active against iNOS compared to nNOS. The values of K_i, n/i and e/i for 2- and 4-trifluoromethylphenyl derivatives **19a**,**b** are, respectively, $K_i \approx 20$ and 40 μM; n/i ≈ 0.06 and 0.01; e/i ≈ 1 and 0.3.



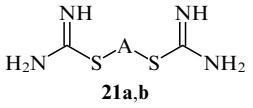
19a,**b**

R = 2-CF_3 (**a**), 4-CF_3 (**b**).



20a,**b**

n = 0 (**a**), 1 (**b**).



21a,**b**

A = $(CH_2)_2$–C$_6$H$_4$–$(CH_2)_2$ (**a**), $(CH_2)_2$–C$_6$H$_4$–$(CH_2)_2$ (**b**).

Table 4. Inhibiting activity of *S*-substituted derivatives of citrulline and thiourea.

Compound	K_i^{iNOS} /μM	Selectivity		Ref.
		n/i	e/i	
15a	4 [a]	0.02	–	64
15b	0.8 [b]	< 0.1	–	33
18a	6 [c]	> 1	> 1	28
18b	0.1	> 1	1	28
	0.02 [d]	> 1	> 1	65
	0.02 [e]	> 10	> 10	66
18c	0.01	> 10	> 10	66
18d	0.2	> 1	> 10	66
21a	0.007	> 1	> 10	66

[a] r-NOS. [b] r-NOS. [c] K_i was calculated from the data published in the study.[31] [d] h-NOS. [e] m-iNOS, r-nNOS, b-eNOS.

Compound **19b** did not inhibit nNOS in a tissue culture (see Table 4).[73]

Amidine **13a** and its analogue **13b** are known as unique inhibitors. Hence, isothiocarbamide derivatives were constructed on the basis of the above compounds.[74] However, compounds **20a**,**b** did not exhibit the expected inhibiting activity. *N*-(3-Aminomethylphenyl)isothiocarbamide (**20a**) was the most active inhibitor (IC_{50}^{r-iNOS} = 23 μM without pronounced selectivity). However, it appeared that the benzyl derivatives of isothiocarbamides (in particular, compound **20b**) serve as h-iNOS stimulators when added simultaneously with ^{14}C-arginine without preincubation with the enzyme.

The introduction of the *N*-amino group into isothiocarbamides (by analogy with aminoguanidine) did not give rise to strong and selective inhibitors. These compounds were many times (> 100) less active than the corresponding *S*-alkylisothiocarbamides. Like aminoguanidine, these compounds inactivate NOS and accelerate autoinactivation of nNOS in the absence of a substrate.[31]

Compounds **21a**,**b** containing two isothiocarbamide groups proved to be active. *S*,*S*′-[1,4-Phenylene(bisethylene)diisothiocarbamide (**21a**) inhibited iNOS even at a nanomolar concentration. A change in the position of the substituents from *para* to *meta* led to the appearance of selectivity, because a substantial decrease in activity of *S*,*S*′-[1,3-phenylene(bisethylene)]diisothiocarbamide (**21b**) toward eNOS was observed ($IC_{50}^{iNOS} \approx 0.05$ μM, n/i ≈ 5, e/i ≈ 200).

Crystallographic study of complexes of *S*-alkylisothiocarbamides (and their selenium analogues) with eNOS demonstrated that not only hydrogen bonds of the conservative amino acids Trp and Glu, which are acceptors of substrates for all isoforms, but also nonpolar interactions in the catalytic pocket of the enzyme play a role in binding to the substrate pocket of the enzyme.[75] Study of the eNOS-inhibiting activity of compounds **19** and **21a**,**b** revealed the topological factors responsible for the affinity of the inhibitors for this constitutive enzyme, which are partially determined by interaction of the second ureide group with the propionate of the pyrrole ring D of the haem.

An increase in the length of the hydrocarbon fragment, which binds two isothiocarbamide residues, to C_{12-14} results in the formation of active NOS inhibitors. Apparently, this is associated with the involvement of new peptide bonds outside the catalytic site and the substrate access channel in a contact with the inhibitor, because bis(isothiocarbamides) (unlike simple *S*-alkylisothiocarbamides) inhibit electron transfer in the reduction domain of NOS.[62] Unfortunately, this class of compounds is of limited pharmacological utility because of toxicity and poor cell membrane permeability.[66]

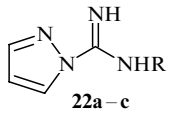
On the whole, both the inhibiting activity of the above-described compounds and their selectivity are determined by the mechanism of the enzyme–inhibitor interaction. The strongest and most selective inhibitors are characterised by either a slow rate of binding to the enzyme or the ability to inactivate the enzyme (suicide inhibitors). The latter (arginine **2b**, ornithine **11a** and isothiocarbamide **18a**) enhance proteolysis of NOS, whereas reversible inhibitors, such as compounds **2a** and **18b**, promote stability of the NOS dimer.[36, 76]

IV. Cyclic analogues of substrate-like inhibitors

The involvement of a guanidine-like group in a cyclic structure opens considerable possibilities for the chemical design of NOS inhibitors recognised by a target enzyme on the principle of similarity with L-arginine. These compounds containing one or two amidine nitrogen atoms in the ring differ somewhat in the mechanism of action.

1. 1*H*-Pyrazole-1-carboxamidines

1*H*-Pyrazole-1-carboxamidines represent a small group of competitive NOS inhibitors containing one of the nitrogen atoms of the guanidine fragment in the heterocycle.[77, 78] 1*H*-Pyrazole-1-carboxamidine (**22a**), which is a very strong but nonselective NOS inhibitor, is a parent compound (Table 5).



22a–c

R = H (**a**), $CH(NH_2)CH_2NH_2$ (**b**), $HNC_6H_4(CH_2)_2NH_2$-3 (**c**).

Table 5. Inhibiting activity of pyrazoles and pyrrolidines.

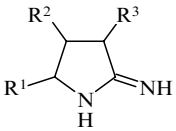
Compound	K_i^{iNOS} /μM	Selectivity		Ref.
		n/i	e/i	
22a	0.7 [a]	~1	~1	78
22b	12	<1	>10	78
23a	5 [b]	<1	>1	56
23b	0.3 [b]	>1	>1	79
23c	0.03	~1	>1	79
23d	0.07	10	400	79
23f	0.15 [b]	>10	>1000	80

[a] m-iNOS, r-nNOS, b-eNOS. [b] K_i was calculated from the data published in the study [31].

The introduction of the methyl group at position 3 or 4 of the pyrazole ring decreases the inhibiting activity by an order of magnitude. On the whole, this class of compounds inhibit nNOS to a greater degree and inhibit eNOS to a smaller degree. N^G-Substituted derivatives, *viz*., *N*-(1,2-diaminoethyl)-1*H*-pyrazole-1-carboxamidine (**22b**) and *N*-(3-aminoethylanilino)-1*H*-pyrazole-1-carboxamidine (**22c**), were found to exhibit high selectivity (n/i ≈ 0.1, e/i ≈ 10).

2. Cyclic amidines

Amidine-containing compounds, primarily, heterocyclic imines and imidazoles, belong to the most well-studied class of cyclic NOS inhibitors. The introduction of aliphatic substituents at positions 4 and 5 of the pyrrolidine ring leads to a substantial increase in the activity and selectivity of new compounds, particularly, of 4-methyl-2-iminopyrrolidine (**23b**) and *trans*-5-ethyl-4-methyl-2-iminopyrrolidine **23c** (see Table 5).



23a–f

$R^1 = R^2 = R^3 = H$ (**a**);
$R^1 = R^3 = H$, $R^2 = Me$ (**b**);
R^1 = *trans*-Et, $R^2 = Me$, $R^3 = H$ (**c**);
R^1 = *cis*-n-C_5H_{11}, R^2 = (+)-Me, $R^3 = H$ (**d**);
R^1 = *cis*-n-C_5H_{11}, R^2 = (+)-CF_3, $R^3 = H$ (**e**);
R^1 = (*R*)-n-C_5H_{11}, R^2 = (*S*)-Me,
R^3 = (*S*)-OH (**f**).

The iNOS-inhibiting activity of pyrrolidine derivatives **23b** and **23c** is two orders of magnitude higher than that of the starting compound **23a** (K_i^{iNOS} < 1 μM). The resolution of racemic mixtures of (+)-4-methyl-*cis*-5-n-pentyl-2-iminopyrrolidine (**23d**) and 4-trifluoromethyl-*cis*-5-n-pentyl-2-iminopyrrolidine (**23e**) into stereoisomers demonstrated that the (+)-*cis* isomers are almost an order of magnitude more selective for iNOS than for eNOS ($K_i \approx 0.1$ μM, n/i ≈ 10, e/i > 100).[79] This is evidence that new stereospecific acceptors were revealed in iNOS. The recently characterised iNOS inhibitor, *viz*., (3*S*,4*S*,5*R*)-3-hydroxy-4-methyl-5-n-pentyl-2-iminopyrrolidine (**23f**), exhibited selectivity (e/i) three times higher than that of compound **23d**. This fact confirmed once again that a search for stereoselective iNOS inhibitors in the class of iminopyrrolidines holds promise.

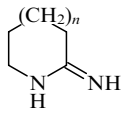
Highly active competitive iNOS inhibitors with K_i < 1 μM were found in a series of heterocyclic imines containing from five to nine carbon atoms in the ring (Table 6). However, these

Table 6. Inhibiting activity of 2-imino-N-containing heterocyclic derivatives.

Compound	K_i^{iNOS} /μM	Selectivity		Ref.
		n/i	e/i	
24a	0.2 [a]	~1	>1	56
24b	0.4	~1	>1	56
25a	0.002 [b]	–	10	81
26a	0.2 [a]	>1	150	82
26b	0.5	10	300	82
26c	0.03	>1	10	82
26d	0.04 [a]	>1	>10	83
26e	0.03 [c]	>1	>10	83
26f	0.1	~2	130	83
26g	0.4	>1	200	83

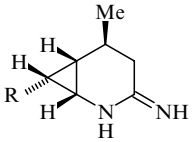
[a] K_i was calculated from the data published in the study [31]. [b] h-NOS. [c] The experimental value of K_i = 0.09 μM.

inhibitors do not exhibit pronounced selectivity. The activity of 2-iminopyrrolidine (**23a**) is *ca*. 25 times lower than that of the six-membered amidine 2-iminopiperidine **24a**. A further increase in the size of the inhibitor molecule leads to a decrease in activity (see Table 6).



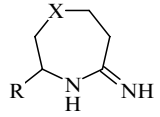
24a,**b**

n = 1 (**a**), 2 (**b**).



25a,**b**

R = Cl (**a**), H (**b**).



26a–**g**

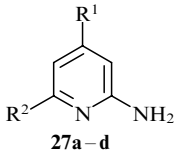
R = MeCH=CHCH$_2$, X = CH$_2$ (**a**);
R = Bu^n, X = O (**b**);
R = (CH$_2$)$_2$NO$_2$, X = CH$_2$ (**c**);
R = CH$_2$CH=CH$_2$, X = O (**d**);
R = CH=CHMe, X = CH$_2$ (**e**);
R = Bu^n, X = CH$_2$ (**f**);
R = 2-Et-2-Bu^n, X = CH$_2$ (**g**).

Fused six-membered amidines were found to have the unique inhibiting properties. In this series, the bicyclic amidine (1*S*,5*S*,6*R*,7*R*)-7-chloro-3-imino-5-methyl-2-azabicyclo[4.1.0]heptane (**25a**) proved to be most active against h-iNOS (see Table 6). Certain fused and spirobicyclic derivatives of 2-iminopiperidine were found to be highly active iNOS inhibitors (IC_{50} < 0.1 μM, e/i > 1).[84] DL-(1*R*,5*S*,6*R*)-3-Imino-5-methyl-2-azabicyclo[4.1.0]-heptane hydrochloride (**25b**) has *in vitro* and *in vivo* very high inhibiting activity (IC_{50}^{h-iNOS} = 0.09, IC_{50}^{m-iNOS} = 0.007 μM, respectively). In experiments on mice treated with lipopolysaccharides, the dose of the agent necessary to decrease the concentration of nitrite anions (the product of NO$^\bullet$ metabolism) in blood plasma was 0.013 mg kg^{-1}, which is almost 1500 times lower than the maximum tolerable level for animals.

The introduction of side substituents and modifications of the ring in hexahydro-2-iminoazepine (**24b**) gave rise to iNOS-selective inhibitors. For example, hexahydro-7-(but-2-enyl)-2-iminoazepine (**26a**) containing the alkenyl substituent at position 7 inhibits iNOS *ca*. 100 times more strongly than eNOS. Due to the presence of the oxygen atom at position 5, the selectivity of hexahydro-7-n-butyl-2-imino-5-oxazepine (**26b**) for iNOS is almost two times higher than that for eNOS, the nNOS-inhibiting activity varying only slightly.[56] The iNOS-inhibiting activity of hexahydro-2-imino-7-(2-nitroethyl)azepine (**26c**) containing the polar nitroethyl group is almost an order of magnitude higher, although the selectivity of this compound is low.

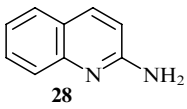
Alkylation at position 7 in hexahydro-2-iminoazepines (see Table 6) results in an increase in activity and selectivity of such inhibitors as hexahydro-7-n-butyl-2-imino-5-oxazepine (**26d**), hexahydro-2-imino-7-propenylazepine (**26e**), hexahydro-7-n-butyl-2-iminoazepine (**26f**) and hexahydro-7-(2-ethyl-2-n-butyl)-2-iminoazepine (**26g**) (K_i < 1 μM, e/i > 100).[83]

The synthesis of analogues of iminopiperidines, *viz*., aminopyridines **27a**–**d**, is a natural continuation of a search for NOS inhibitors among heterocyclic imines.



27a–**d**

R^1 = Me: R^2 = H (**a**), Bu^t (**b**);
R^1 = H: R^2 = Ac (**c**),
(CH$_2$)$_3$–N NH (**d**).



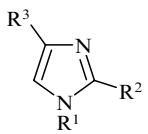
28

Later on, one of the first inhibitors belonging to this class, *viz*., 2-amino-4-methylpyridine (**27a**)[85] (K_i are 55, 65 and 98 μM for h-iNOS, h-nNOS and h-eNOS, respectively), has been used as a competitive radioligand to estimate the affinity and selectivity of other NOS inhibitors.[31] However, neither compound **27a** nor other mono- and disubstituted alkyl derivatives of 2-aminopyridine exhibited pronounced selectivity for the inducible enzyme (n/i > 1, e/i > 1). Of these compounds, 2-amino-6-*tert*-butyl-4-methylpyridine (**27b**) is the strongest iNOS inhibitor (IC_{50} < 1 μM). 2-Amino-6-(4-substituted)phenylpyridines (for example, **27c**) did not have iNOS-inhibiting activity. However, arylidenepiperazine-bridged compound **27d** appeared to be an h-nNOS strong inhibitor (IC_{50} < 1 μM), the selectivity being low (e/n > 1). Compound **27d** exhibited the highest selectivity and inhibited h-nNOS six times more strongly than h-eNOS (IC_{50}^{h-nNOS} = 0.14 μM).[86] 2-Aminopyridines fused with five- or six-membered saturated and unsaturated rings are active iNOS inhibitors (IC_{50} < 10 μM) without pronounced selectivity, compounds containing unsaturated rings being more active. 2-Aminoquinoline **28** is the most active iNOS inhibitor (IC_{50} ≈ 2 μM).[87]

3. Imidazoles and nitroindazoles

Imidazole derivatives can be considered as cyclic analogues of carboxamidines containing both amidine nitrogen atoms in the heterocycle. Their reactions with the enzyme are targeted primarily to the haem. Imidazoles bind to the enzyme as the sixth ligand of the hexacoordinate iron atom in the haem, *i.e*., analogously to CO, NO$^\bullet$, O_2 and CN^-. This property was well studied for cytochrome P-450, which is closely similar to NOS.[88]

Inhibition of NOS by imidazoles occurs reversibly. However, depending on the nature of the substituents and the NOS source, imidazoles compete with BH_4 and/or L-arginine for binding to the catalytic site. For example, 1-phenylimidazole (**29a**) (IC_{50}^{h-iNOS} ≈ 40 μM), which is one of the strongest iNOS inhibitors, noncompetitively (with respect to L-arginine) binds to m-iNOS and competitively binds to b-eNOS (Table 7).



29a–**e**

R^1 = Ph, R^2 = R^3 = H (**a**);
R^1 = 2-CF$_3$C$_6$H$_4$, R^2 = R^3 = H (**b**);
R^1 = (CH$_2$)$_4$CH(NH$_2$)CO$_2$H, R^2 = NH$_2$, R^3 = H (**c**);
R^1 = H, R^2 = NH$_2$(NH)CNHCH$_2$, R^3 = H (**d**);
R^1 = (CH$_2$)$_n$CH(NH$_2$)CO$_2$H, R^2 = R^3 = H, n = 3 or 5 (**e**).

These differences in the inhibition mechanisms makes it difficult to interpret the inhibition constants determined by spectroscopic or kinetic methods.

Modifications of the substituent at the nitrogen atom afford inhibitors active against nNOS. However, frequently used 1-(2-

Table 7. Inhibiting activity of imidazole and 7-nitroindazole derivatives.

Compound	K_i^{iNOS} /μM	Selectivity		Ref.
		n/i	e/i	
29a	35 [a]	~10	~20	10
29b	30 [a, b]	1	>10	89, 90
29c	30 [a, d]	<1	<1	91
29d	10	<1	>1	92
31b	0.03 [a, e]	>1	1000	93
33	1.6 [a, f]	0.1	0.5	94

[a] IK_{50}. [b] r-iNOS, m-nNOS, b-eNOS. [c] r-nNOS, e-hNOS. [d] r-iNOS, r-nNOS, h-eNOS. [e] h-iNOS, crude cell lysate. [f] m-iNOS, b-nNOS, b-eNOS.

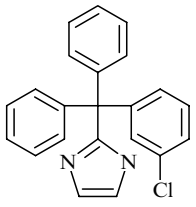
trifluoromethylphenyl)imidazole (**29b**) did not exhibit considerable activity and selectivity in *in vitro* experiments (see Table 7), although it caused a decrease in pain sensitivity of animals to formalin, which reflects its nNOS-inhibiting ability. Since imidazole derivatives containing substituents at positions 2 and 4 have lower inhibiting activity (IC_{50} > 1000 μM),[95] attempts to design highly efficient inhibitors belonging to the class of 1,2-disubstituted imidazoles failed. It is believed that compounds with this structure may be useful tools for revealing the characteristic features of the guanidine–oxygen- and BH_4-binding regions of NOS.[96]

An increase in the length of the aliphatic bridge between the imidazole and the amino acid group made it possible to prepare active compounds.[92] Inhibitors **29e** containing an odd number of methylene groups are more selective for nNOS (K_i^{nNOS} = 2 μM for both compounds). Compounds containing an even number of methylene groups are less active.

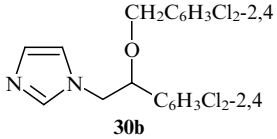
Crystallographic studies demonstrated that two imidazole molecules can simultaneously bind to the oxidative domain of the m-iNOS monomer: one molecule is located in the catalytic site as the sixth ligand of the haem iron atom, and another molecule is located in the guanidine-binding pocket with Glu371.[22] In this connection, guanidine-substituted imidazoles were synthesised. However, most agents appeared to be poorly active, if at all. Among these compounds, imidazole derivative **29d** is the strongest inhibitor (against iNOS; $IC_{50} \approx 140$ μM).[97]

Attempts were made to enhance the inhibiting activity of imidazoles by including an amino acid residue into molecules of these inhibitors. 2-Amino-5-(imidazol-1-yl)pentanoic acid (**29c**) has the highest activity (see Table 7).

Antifungal clotrimazole (**30a**) and miconazole (**30b**), which inhibit nNOS competitively with CaM, belong to imidazoles containing bulky substituents.[98]



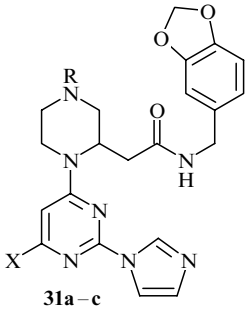
30a



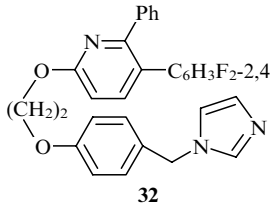
30b

In addition, these drugs *in vitro* inhibit dimerisation of the oxidative domain of iNOS. In RAW264.7 cells, clotrimazole (50 μM) also blocks the formation of dimeric iNOS,[99] unlike substrate-like inhibitors, in the presence of which the fraction of dimeric iNOS increases from 50% to 90%.[18, 69]

Powerful inhibitors with bulky substituents were found by screening 8649 compounds prepared by combinatorial chemistry based on nitrogen-containing heterocycles.[93] Of 53 compounds inhibiting iNOS by 60% at 200 μM, three compounds, **31a–c**, which have IC_{50}^{iNOS} < 1 μM in a cell test system, were chosen.

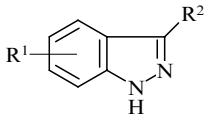


31a–c



32

R = H, X = Cl (**a**); R = CO_2Me, X = H (**b**);
R = CO_2Et, X = H (**c**).



33a–e

R^1 = 7-NO_2: R^2 = H (**a**), Br (**b**);
R^1 = 6-NO_2, R^2 = H (**c**);
R^1 = R^2 = H (**d**);
R^1 = 5-NO_2, R^2 = H (**e**).

Compound **31b** was a highly efficient nNOS inhibitor not only in a cell culture,[100] but also in animals treated with a lipopolysaccharide (ID_{50} = 1.2 mg kg^{-1})§ (based on the criterion of a decrease in the nitrite level in rat blood plasma). Crystallographic analysis of the complex of the oxidative domain of iNOS with compound **31b** demonstrated that free nitrogen of the imidazole group is coordinated to the haem iron atom in the sixth axial position, and the structure of the guanidine-binding sequence involving Glu371 is destroyed under the action of the adjacent imidazole, pyrimidine and piperazine rings. Inhibitors belonging to this series of compounds do not suppress activity of dimeric NOS. However, inhibitors were found among imidazole-containing compounds.

3-(2,4-Difluorophenyl)-6-{2-[4-(1*H*-imidazol-1-ylmethyl)phenoxy]ethoxy}-2-phenylpyridine (**32**) inhibits the NO• synthesis in activated macrophages with IC_{50} = 0.08 μM and have the therapeutic effect in experimental arthritis models.[101]

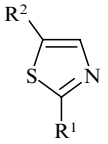
The inhibition mechanism involving the interaction with the haem was observed for compounds of the nitroindazole series. The most well-known agent, *viz*., 7-nitroindazole **33a**, is a competitive inhibitor of iNOS (against L-arginine and BH_4) and eNOS (against BH_4). This is associated with the fact that compound **33a**, like its analogue, *viz*., 3-bromo-7-nitroindazole (**33b**) (IC_{50}^{r-iNOS} = 0.29 μM, n/I < 1, e/I > 1),[102] contacts with one of the propionate groups of the haem, thus suppressing its interaction with BH_4.[62]

Nitroindazoles **33c** (K_i^{b-nNOS} = 40 μM) and **33a** were the strongest b-nNOS inhibitors, whereas the inhibiting activities of indazole (**33d**) and 5-nitroindazole (**33e**) were very weak (IC_{50} > 1000 μM).[103] However, according to the results of the study,[104] compounds **33c**,**e** are virtually identical in the r-nNOS-inhibiting activity. The selectivity of 7-nitroindazole **33a** for nNOS, which is manifested *in vivo*, is apparently associated with its cell membrane permeability and metabolism.[14]

4. Cyclic analogues of isothioureas

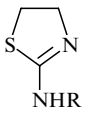
Cyclisation of *S*-aminoalkylisothioureas affords the corresponding S,N-heterocycles with structures isosteric with the guanidine group of L-arginine.[105, 106] Although thiazole **34a** is a very weak inhibitor of all NOS isoforms (K_i > 200 μM), the introduction of an amino group giving rise to 2-aminothiazole (**34b**) increases the inhibiting activity against iNOS by a factor of *ca*. 5 (Table 8).

§ ID_{50} is the dose at which the rate of the NO• synthesis is half that observed for an intact sample.



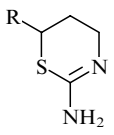
34a–d

$R^1 = R^2 = H$ (**a**);
$R^1 = NH_2$: $R^2 = H$ (**b**), Me (**c**);
$R^1 = NH(CH_2)_3Cly$, $R^2 = H$ (**d**).



35a,b

R = H (**a**);
$(CH_2)_3$Ala (**b**).



36a,b

R = H (**a**);
Me (**b**).

Table 8. Inhibiting activity of cyclic analogues of isothioureas.

Compound	K_i^{iNOS} /μM	Selectivity		Ref.
		n/i	e/i	
34b	50 [a]	< 1	< 1	61
35a	0.3	2	1	61
35b	8 [b,c]	< 1	< 1	107
36a	0.6 [d]	1	2	56
36b	0.004 [e]	> 10	> 10	65
39	9 [f]	1	0.3	108
40	2	20	4	108
41	2	25	1	108
42	0.8 [g]	> 1	> 1	109

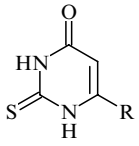
[a] h-NOS. [b] IC_{50}. [c] r-iNOS, r-nNOS, h-eNOS. [d] K_i was calculated from the data published in the study.[31] [e] m-iNOS, r-nNOS, b-eNOS. [f] [Arg] = 5 μM, m-iNOS, m-nNOS, b-eNOS. [g] h-NOS.

2-Amino-5-methylthiazole (**34c**) ($IC_{50} \approx 12$ μM) is even more active.[61] However, these inhibitors exhibited selectivity for none of the NOS isoforms. *N*(5)-(Thiazolin-2-yl)-L-ornithine (**35b**) prepared by modifying the amino group in 2-amino-2-thiazoline (**35a**) is an active eNOS inhibitor ($IC_{50} \approx 1$ μM). At the same time, *N*(5)-(thiazol-2-yl)-L-ornithine (**34d**) is a weak inhibitor.[107]

The activity and selectivity of 2-amino-5,6-dihydro-4*H*-1,3-thiazine (**36a**) synthesised by cyclisation of *S*-aminopropylisothiourea exhibits are similar to those of compound **35a** (see Table 8). The replacement at position 6 leads to a substantial increase in activity and selectivity of the derivatives (as in the case of N-containing heterocyclic imines) (see Table 6). For example, 2-amino-6-methyl-5,6-dihydro-4*H*-1,3-thiazine (**36b**) inhibits iNOS almost 150 times more strongly than thiazine **36a**.

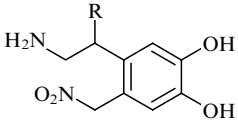
5. Other inhibitors

The NOS-inhibiting activity was found in a series of antithyroid agents belonging to thiouracils. 6-Methyl- (**37a**) and 6-n-propyl-2-thiouracils (**37b**) exhibited selectivity for r-nNOS inhibition. At a concentration of 500 μM, compound **37a** inhibited the synthesis of L-citrulline by the enzyme isoforms m-iNOS, r-nNOS and b-eNOS by ≈ 20%, 80% and 40%, respectively.[110] The inhibition occurred competitively and reversibly, suppression of BH_4-dependent dimerisation of nNOS playing a certain role in this effect.[111]



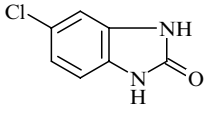
37a,b

R = Me (**a**), Pr^n (**b**).

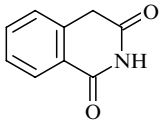


38a,b

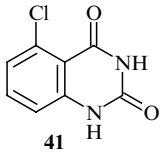
R = H (**a**), OH (**b**).



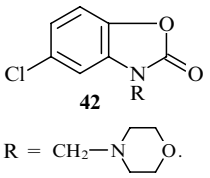
39



40



41

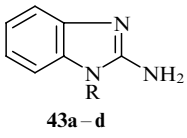


42

The introduction of the nitro group in dopamine and noradrenaline gave rise to the corresponding nitro derivatives, *viz*., 4-(2-aminoethyl)-5-nitromethylpyrocatechol (**38a**) and 4-(2-aminoethyl-1-hydroxy)-5-nitromethylpyrocatechol (**38b**). These compounds inhibited NOS two orders of magnitude more strongly ($K_i \approx 50$ μM) than the starting catecholamines. The inhibition occurred reversibly and competitively with L-arginine and BH_4; in addition, slight selectivity for r-nNOS, was observed, which may be attributed to suppression of enzyme dimerisation.[112] These results suggest that the nitro group plays a great role in inhibitor–NOS complexation.

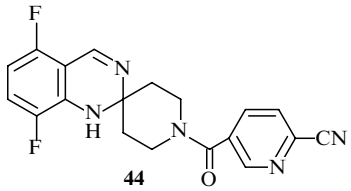
Screening of a chemical library revealed a series of promising bicyclic NOS inhibitors. Thus, IC_{50}^{m-iNOS} for 5-chloro-1,3-dihydro-2*H*-benzimidazol-2-one (**39**), (2*H*,4*H*)-isoquinoline-1,3-dione (**40**) and 5-chloro-(1*H*,3*H*)-quinazolone-2,4-dione (**41**) were < 10 μM. The selectivity of compounds **39** and **41** for r-nNOS and b-eNOS (n/i) is > 10 and > 1, respectively. In a series of benzoxazolones, 6-chloro-*N*-morpholinomethylbenzoxazolone (**42**) appeared to be an active h-iNOS inhibitor, which, however, did not exhibit noticeable selectivity (see Table 8).

It is known [113] that substituted 2-aminobenzimidazoles **43a–d** have NOS-inhibiting activity. 2-Aminobenzimidazole (**43a**) competitively inhibited iNOS, the selectivity for eNOS being very low ($IC_{50}^{h-iNOS} = 25$ μM, n/i ≈ 3, e/i > 5). 2-Amino-1-aminomethylbenzimidazole (**43b**) is more selective for h-nNOS ($IC_{50}^{h-nNOS} \approx 6$ μM, n/i < 0.1, n/e < 0.1). 1-Ethyl- (**43d**) and 2-amino-1-isopropylaminobenzimidazoles (**43c**) are inactive.[113]



43a–d

R = H (**a**), CH_2NH_2 (**b**),
Pr^iNH (**c**), Et (**d**).



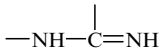
44

Recently, compounds belonging to the class of 1,2-dihydroquinazolineamines and their spirocyclic derivatives were found to have inhibiting activity against iNOS.[114] For example, 1-(6-cyano-3-pyridylcarbonyl)-5′,8′-difluorospiro[piperidine-4,2′(1′*H*)-quinazoline]-4′-amine hydrochloride (**44**) showed high efficiency both *in vitro* and *in vivo* ($IC_{50}^{h-iNOS} = 0.04$ μM, n/i > 10, e/i > 1000).

V. Conclusion

Based on the above data, the following physicochemical features responsible for the efficiency of NOS inhibitors, which interact with the catalytic site of the enzyme, can be noted.

1. The guanidine, carboxamidine or isothiocarbamide groups in linear or cyclic structures



provide hydrogen bonding with Glu and/or the formation of a coordination bond with the haem Fe atom.

2. Small hydrophobic substituents at the guanidine-like group (alkyl, phenyl or thienyl), which can form additional bonds with haem-proximal peptide regions, should be present.

3. The chemical activity of a guanidine-like group as a pseudosubstrate can have a determining effect on both the selectivity and inhibiting activity of substrate-like compounds.

4. In addition to these factors, such properties of compounds as suppression of superoxide synthase activity of the enzyme, regulation of NOS dimerisation and inactivation of active oxygen forms produced by NOS can facilitate efficiency of *in vivo* NOS inhibition.[115]

One would expect that the next step in the chemical synthesis and design of pharmacological agents modifying the enzymatic $NO^{\bullet}$ production will be aimed at preparing compounds that combine the following functions: specificity for NOS isoforms, antioxidant activity and specificity for accumulation in subcellular targets (caveoles, synapses, mitochondria, *etc.*).

We thank Prof. T M Roshchina, Prof. F A Tpofimov and Dr. V Yu Kovtun for helpful discussion and valuable advice. We also acknowledge the designers of the molbiol.ru site for the information support.

This review has been written with the financial support of the Russian Foundation for Basic Research (Project Nos 03-04-48349 and 04-04-48218), the Administration of the Kaluzhskaya Region (Project No. 04-04-97238) and the International Science and Technology Centre (ISTC, Project No. 779B).

References

1. L J Ignarro, K S Wood, R E Byms *Circulation* **74** II-287 (1986)
2. R M J Palmer, A G Ferrige, S Moncada *Nature (London)* **327** 524 (1987)
3. S Ya Proskuryakov, A G Konoplyannikov, A I Ivannikov, V G Skvortsov *Usp. Sovr. Biol.* **119** 380 (1999)
4. S Ya Proskuryakov, A I Ivannikov, V G Skvortsov, S F Biketov *Immunologiya* **4** 9 (2000)
5. S Ya Proskuryakov, A G Konoplyannikov, A I Ivannikov, V G Skvortsov, A F Tsyb *Vopr. Onkolog.* **47** 257 (2001)
6. S Ya Proskuryakov, V L Gabai, A G Konoplyannikov *Biokhimiya* **67** 467 (2002) [a]
7. M N Muscara, J L Wallace *Am. J. Physiol.* **276** G1313 (1999)
8. A J Hobbs, A Higgs, S Moncada *Ann. Rev. Pharmacol. Toxicol.* **39** 191 (1999)
9. V G Granik, S Yu Ryabova, N B Grigoriev *Usp. Khim.* **66** 792 (1997) [*Russ. Chem. Rev.* **66** 717 (1997)]
10. L Salerno, V Sorrenti, C Di Giacomo, G Romeo, M A Siracusa *Curr. Pharm. Des.* **8** 177 (2002)
11. E W J A Albrecht, C A Stegeman, P Heeringa, R H Henning, H van Goor *J. Pathol.* **199** 8 (2003)
12. D W Reif, D J McCarthy, E Gregan, J E Macdonald *Free Radical Biol. Med.* **28** 1470 (2000)
13. W K Alderton, C E Cooper, R G Knowles *Biochem. J.* **357** 593 (2001)
14. A C F Gorren, B Mayer *Curr. Drug Metab.* **3** 133 (2002)
15. J L Zweier, A Samouilov, P Kuppusamy *Biochim. Biophys. Acta* **1411** 250 (1999)
16. D K Ghosh, J C Salerno *Front. Biosci.* **8** D193 (2003)
17. H Li, C S Raman, C B Glaser, E Blasko, T A Young, J F Parkinson, M Whitlow, T L Poulos *J. Biol. Chem.* **274** 21276 (1999)
18. Q A Albakri, D J Stuehr *J. Biol. Chem.* **271** 5414 (1996)
19. B M List, B Klosch, C Volker, A C F Gorren, W C Sessa, E R Werner, W R Kukovetz, K Schmidt, B Mayer *Biochem. J.* **323** 159 (1997)
20. K Panda, R J Rosenfeld, S Ghosh, A L Meade, E D Getzoff, D J Stuehr *J. Biol. Chem.* **277** 31020 (2002)
21. T O Fischmann, A Hruza, X D Niu, J D Fossetta, C A Lunn, E Dolphin, A J Prongay, P Reichert, D J Lundell, S K Narula, P C Weber *Nat. Struct. Biol.* **6** 233 (1999)
22. Y Lee, M A Marletta, P Martasek, L J Roman, B S S Masters, R B Silverman *Bioorg. Med. Chem.* **7** 1097 (1999)
23. R Fedorov, E Hartman, D K Ghosh, I Schlichting *J. Biol. Chem.* **278** 45818 (2003)
24. B R Babu, C Frey, O W Griffith *J. Biol. Chem.* **274** 25218 (1999)
25. B C Fan, J L Wang, D J Stuehr, D L Rousseau *Biochemistry* **36** 12660 (1997)
26. N C Gerber, I Rodriguez-Crespo, C R Nishida, P R O de Montellano *J. Biol. Chem.* **272** 6285 (1997)
27. L E Bretscher, H Y Li, T L Poulos, O W Griffith *J. Biol. Chem.* **278** 46789 (2003)
28. S Moncada, A Higgs, K Furchgott *Pharmacol. Rev.* **49** 137 (1997)
29. P E Chabrier, M Auguet, B Spinnewyn, S Auvin, S Cornet, C Demerle-Pallardy, C Guilmard-Favre, J G Marin, B Pignol, V Gillard-Roubert, C Roussillotc-Harnet, J Schulz, I Viossat, D Bigg, S Moncada *Proc. Natl. Acad. Sci. USA* **96** 10824 (1999)
30. K Narayanan, L Spack, K Mcmillan, R G Kilbourn, M A Hayward, B S S Masters, O W Griffith *J. Biol. Chem.* **270** 11103 (1995)
31. R Boer, W-R Ulrich, T Klein, B Mirau, S Haas, I Baur *Mol. Pharmacol.* **58** 1026 (2000)
32. D J Wolf, D S Gauld, M J Neulander, G Southan *J. Exp. Pharmacol. Ther.* **283** 265 (1977)
33. J L Collins, B G Shearer, J A Oplinger, S L Lee, E P Garvey, M Salter, C Duffy, T C Burnette, E S Furfine *J. Med. Chem.* **41** 2858 (1998)
34. H Q Zhang, W Fast, M A Marletta, P Martasek, R B Silverman *J. Med. Chem.* **40** 3869 (1997)
35. M Dambrova, O Kirjanova, L Baumane, E Liepinsh, L Zvejniece, R Muceniece, I Kalvinsh, J E S Wikberg *J. Physiol. Pharmacol.* **54** 339 (2003)
36. S Noguchi, S Jianmongkol, A T Bender, Y Kamada, D R Demady, Y Osawa *J. Biol. Chem.* **275** 2376 (2000)
37. E S Furfine, M F Harmon, J E Paith, E P Garvey *Biochemistry* **32** 8512 (1993)
38. D W Reif, S A Mccreedy *Arch. Biochem. Biophys.* **320** 170 (1995)
39. J L Vuletich, E R Lowe, S Jianmongkol, Y Kamada, U M Kent, A T Bender, D R Demady, P F Hollenberg, Y Osawa *Mol. Pharmacol.* **62** 110 (2002)
40. P L Feldman, O W Griffith, H Hong, D J Stuehr *J. Med. Chem.* **36** 491 (1993)
41. N M Olken, M A Marletta *Biochemistry* **32** 9677 (1993)
42. N M Olken, Y Osawa, M A Marletta *Biochemistry* **33** 14784 (1994)
43. A J Cardounel, J L Zweier *J. Biol. Chem.* **277** 33995 (2002)
44. N M Olken, M A Marletta *J. Med. Chem.* **35** 1137 (1992)
45. C Mitaka, Y Hirata, K Yokoyama, T Imai *J. Crit. Care* **16** 17 (2001)
46. D Fishlock, B Perdicakis, H J Montgomery, J G Guillemette, E Jervis, G A Lajoie *Bioorg. Med. Chem.* **11** 869 (2003)
47. V G Granik, N B Grigoriev *Izv. Akad. Nauk, Ser. Khim.* 1286 (2002) [b]
48. D R Demady, S Jianmongkol, J L Vuletich, A T Bender, Y Osawa *Mol. Pharmacol.* **59** 24 (2001)
49. R B Silverman, H Huang, M A Marletta, P Martasek *J. Med. Chem.* **40** 2813 (1997)
50. H Huang, P Martasek, L J Roman, B S S Masters, R B Silverman *J. Med. Chem.* **42** 3147 (1999)
51. J M Hah, L J Roman, P Martasek, R B Silverman *J. Med. Chem.* **44** 2667 (2001)
52. J M Hah, P Martasek, L J Roman, R B Silverman *J. Med. Chem.* **46** 1661 (2003)
53. M Cowart, E A Kowaluk, J F Daanen, K L Kohlhaas, K M Alexander, F L Wagenaar, J F Kerwin *J. Med. Chem.* **41** 2636 (1998)
54. B R Babu, O W Griffith *J. Biol. Chem.* **273** 8882 (1998)
55. W M Moore, R K Webber, K F Fok, G M Jerome, J R Connor, P T Manning, P S Wyatt, T P Misko, F S Tjoeng, M G Currie *J. Med. Chem.* **39** 669 (1996)
56. E A Hallinan, S Tsymbalov, P M Finnegan, W M Moore, G M Jerome, M G Currie, B S Pitzele *J. Med. Chem.* **41** 775 (1998)
57. R J Young, R M Beams, K Carter, H A R Clark, D M Coe, C L Chambers, P I Davies, J Dawson, M J Drysdale, K W Franzman, C French, S T Hodgson, H F Hodson, S Kleanthous, P Rider, D Sanders, D A Sawyer, K J Scott, B G Shearer, R Stocker, S Smith, M C Tackley, R G Knowles *Bioorg. Med. Chem. Lett.* **10** 597 (2000)
58. R J Young, W K Alderton, P J Beswick, D Brown, C L Chambers, M Chandler, C L Craig, J Dawson, S J Faulkes, S T Hodgson, S Kleanthous, R Stocker, M C Tackley, R G Knowles, in *ASC Meeting (Abstracts of Reports), Chicago, IL, 2001* p. 53

59. E A Hallinan, S W Kramer, S C Houdek, W M Moore, G M Jerome, D P Spangler, A M Stevens, H S Shieh, P T Manning, B S Pitzele *Org. Biomol. Chem.* **1** 3527 (2003)
60. G J Southan, C Szabo, M P Connor, A L Salzman, C Thiemermann *Eur. J. Pharmacol.* **291** 311 (1995)
61. E P Garvey, J A Oplinger, E S Furfine, R J Kiff, F Laszlo, B J R Whittle, R G Knowles *J. Biol. Chem.* **272** 4959 (1997)
62. C S Raman, H Li, P Martasek, G Southan, B S Masters, T L Poulos *Biochemistry* **40** 13448 (2001)
63. E A Hallinan, S Tsymbalov, C R Dorn, B S Pitzele, D W Hansen, W M Moore, G M Jerome, J R Connor, L F Branson, D L Widomski, Y Zhang, M G Currie, P T Manning *J. Med. Chem.* **45** 1686 (2002)
64. C Frey, K Narayanan, K Mcmillan, L Spack, S S Gross, B S Masters, O W Griffith *J. Biol. Chem.* **269** 26083 (1994)
65. M Nakane, V Klinghofer, J E Kuk, J L Donnelly, G P Budzik, J S Pollock, F Basha, G W Carter *Mol. Pharmacol.* **47** 831 (1995)
66. E P Garvey, J A Oplinger, G J Tanoury, P A Sherman, M Fowler, S Marshall, M F Harmon, J E Paith, E S Furfine *J. Biol. Chem.* **269** 26669 (1994)
67. N Kobayashi, T Higuchi, Y Urano, K Kikuchi, M Hirobe, T Nagano *Biol. Pharm. Bull.* **22** 936 (1999)
68. N Sennequier, D J Stuehr *Biochemistry* **35** 5883 (1996)
69. H Ruetten, C Thiemermann *Biochem. Biophys. Res. Commun.* **225** 525 (1996)
70. D Doherty, R Shapiro, W T Burnett *J. Am. Chem. Soc.* **79** 5667 (1957)
71. G J Southan, B Zingarelli, M O'Connor, A L Salzman, C Szabo *Br. J. Pharmacol.* **117** 619 (1996)
72. N C Stratman, G J Fici, V H Sethy *Life Sci.* **59** 945 (1996)
73. B G Shearer, S L Lee, J A Oplinger, L W Frick, E P Garvey, E S Furfine *J. Med. Chem.* **40** 1901 (1997)
74. C L Goodyer, E C Chinje, M Jaffar, I J Stratford, M D Threadgill *Bioorg. Med. Chem.* **11** 4189 (2003)
75. H Y Li, C S Raman, P Martasek, V Kral, B S S Masters, T L Poulos *J. Inorg. Biochem.* **81** 133 (2000)
76. Y Osawa, E R Lowe, A C Everett, A Y Dunbar, S S Billecke *J. Pharmacol. Exp. Ther.* **304** 493 (2003)
77. G J Southan, D Gauld, A Lubeskie, B Zingarelli, S Cuzzocrea, A L Salzman, C Szabo, D J Wolff *Biochem. Pharmacol.* **54** 409 (1997)
78. Y Lee, P Martasek, L J Roman, R B Silverman *Bioorg. Med. Chem. Lett.* **10** 2771 (2000)
79. T J Hagen, A A Bergmanis, S W Kramer, K F Fok, A E Schmelzer, B S Pitzele, L Swenton, G M Jerome, C M Kornmeier, W M Moore, L F Branson, J R Connor, P T Manning, M G Currie, E A Hallinan *J. Med. Chem.* **41** 3675 (1998)
80. S Tsymbalov, T J Hagen, W M Moore, G M Jerome, J R Connor, P T Manning, B S Pitzele, E A Hallinan *Bioorg. Med. Chem. Lett.* **12** 3337 (2002)
81. M Naka, T Nanbu, K Kobayashi, Y Kamanaka, M Komeno, R Yanase, T Fukutomi, S Fujimura, H G Seo, N Fujiwara, S Ohuchida, K Suzuki, K Kondo, N Taniguchi *Biochem. Biophys. Res. Commun.* **270** 663 (2000)
82. A E Moormann, S Metz, M V Toth, W M Moore, G Jerome, C Kornmeier, P Manning, D W Hansen, B S Pitzele, R K Webber *Bioorg. Med. Chem. Lett.* **11** 2651 (2001)
83. D W Hansen, K B Peterson, M Trivedi, S W Kramer, R K Webber, F S Tjoeng, W M Moore, G M Jerome, C M Kornmeier, P T Manning, J R Connor, T P Misko, M G Currie, B S Pitzele *J. Med. Chem.* **41** 1361 (1998)
84. Y Kawanaka, K Kobayashi, S Kusuda, T Tatsumi, M Murota, T Nishiyama, K Hisaichi, A Fujii, K Hirai, M Naka, M Komeno, Y Odagaki, H Nakai, M Toda *Bioorg. Med. Chem.* **11** 1723 (2003)
85. W S Faraci, A A Nagel, K A Verdries, L A Vincent, H Xu, L E Nichols, J M Labasi, E D Salter, E R Pettipher *Br. J. Pharmacol.* **119** 1101 (1996)
86. J A Lowe, W M Qian, R A Volkmann, S Heck, J Nowakowski, R Nelson, C Nolan, D Liston, K Ward S Zorn, C Johnson, M Vanase,W S Faraci, K A Verdries, J Baxter, S Doran, M Sanders, M Ashton, P Whittle, M Stefaniak *Bioorg. Med. Chem. Lett.* **9** 2569 (1999)
87. W K Hagmann, C G Caldwell, P Chen, P L Durette, C K Esser, T J Lanza, I E Kopka, R Guthikonda, S K Shah, M MacCoss, R M Chabin, D Fletcher, S K Grant, B G Green, J L Humes, T M Kelly, S Luell, R Meurer, V Moore, S G Pacholok, T Pavia, H R Williams, K K Wong *Bioorg. Med. Chem. Lett.* **10** 1975 (2000)
88. K A White, M A Marletta *Biochemistry* **31** 6627 (1992)
89. R L S Handy, P K Moore *Life Sci.* **60** PL389 (1997)
90. R L S Handy, H P Wallace, Z A Gaffen, K J Whitehead, P K Moore *Br. J. Pharmacol.* **116** 2349 (1995)
91. S Ulhaq, E C Chinje, M A Naylor, M Jaffar, L J Stratford, M D Threadgill *Bioorg. Med. Chem.* **6** 2139 (1998)
92. Y Lee, P Martasek, J Roman, B S S Masters, R B Silverman *Bioorg. Med. Chem.* **7** 1941 (1999)
93. K McMillan, M Adler, D S Auld, J J Baldwin, E Blasko, L J Browne, D Chelsky, D Davey, R E Dolle, K A Eagen, S Erickson, R I Feldman, C B Glaser, C Mallari, M M Morrissey, M H Ohlmeyer, G Pan, J F Parkinson, G B Phillips, M A Polokoff, N H Sigal, R Vergona, M Whitlow, T A Young, J J Devlin *Proc. Natl. Acad. Sci. USA* **97** 1506 (2000)
94. D J Wolff, A Lubeskie, S Umansky *Arch. Biochem. Biophys.* **314** 360 (1994)
95. R M Chabin, E McCauley, J R Calaycay, T M Kelly, K L MacNaul, C Wolfe, N I Hutchinson, S Madhusudanaraju, J A Schmidt, J W Kozarich, K K Wong *Biochemistry* **35** 9567 (1996)
96. V Berka, G Palmer, P F Chen, A L Tsai *Biochemistry* **37** 6136 (1998)
97. R N Atkinson, S B King *Bioorg. Med. Chem. Lett.* **9** 2953 (1999)
98. D J Wolff, G A Datto, R A Samatovicz *J. Biol. Chem.* **268** 9430 (1993)
99. N Sennequier, D Wolan, D J Stuer *J. Biol. Chem.* **274** 930 (1999)
100. H-J Habisch, A C F Gorren, H Liang, R C Venema, J F Parkinson, K Schmidt, B Mayer *Mol. Pharm.* **63** 682 (2003)
101. M Ohtsuka, F Konno, H Honda, T Oikawa, M Ishikawa, N Iwase, K Isomae, F Ishii, H Hemmi, S Sato *J. Pharmacol. Exp. Ther.* **303** 52 (2002)
102. P A Bland-Ward, P K Moore *Life Sci.* **57** PL131 (1995)
103. D J Wolff, B J Gribin *Arch. Biochem. Biophys.* **311** 300 (1994)
104. R C Babbedge, P A Blandward, S L Hart, P K Moore *Br. J. Pharmacol.* **110** 225 (1993)
105. A G Tarasenko, E Ya Graevskii, M M Konstantinova, A A Mandrugin, I V Nekrasova, V M Fedoseev *Dokl. Akad. Nauk SSSR* **187** 203 (1969) [c]
106. G V Dontsova, M M Konstantinova, A A Mandrugin, O N Rakhmanina, V M Fedoseev, Yu V Shlykov *Khim.-Farm. Zh.* (3) 306 (1986) [d]
107. S Ulhaq, E C Chinje, M A Naylor, M Jaffar, I J Stratford, M D Threadgill *Bioorg. Med. Chem.* **7** 1787 (1999)
108. Y Kita, M Muramoto, A Fujikawa, T Yamazaki, Y Notsu, S Nishimura *J. Pharm. Pharmacol.* **54** 1141 (2002)
109. K Shankaran, K L Donelly, S K Shah, J L Humes, S G Pacholok, S K Grant, B G Gree, M Maccoss *Bioorg. Med. Chem. Lett.* **7** 2887 (1997)
110. A Palumbo, M d'Ischia *Biochem. Biophys. Res. Commun.* **282** 793 (2001)
111. A Palumbo, M d'Ischia, F A Cioffi *FEBS Lett.* **485** 109 (2000)
112. A Palumbo, G Astarita, M d'Ischia *Biochem J.* **356** 105 (2001)
113. P Hamley, A C Tinker *Bioorg. Med. Chem. Lett.* **5** 1573 (1995)
114. A C Tinker, H G Beaton, N Boughton-Smith, T R Cook, S L Cooper, L Fraser-Rae, K Hallam, P Hamley, T McInally, D J Nicholls, A D Pimm, A V Wallace *J. Med. Chem.* **46** 913 (2003)
115. S Ya Proskuryakov, A G Konoplyannikov, V G Skvortsov, A A Mandrugin, V M Fedoseev *Biokhimiya* **70** 82 (2005) [a]

[a] — *Biochemistry (Moscow) (Engl. Transl.)*
[b] — *Russ. Chem. Bull., Int. Ed. (Engl. Transl.)*
[c] — *Dokl. Chem. (Engl. Transl.)*
[d] — *Pharm.-Chem. J. (Engl. Transl.)*