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N-Substituted acetamidines and 2-methylimidazole derivatives as selective inhibitors of neuronal nitric oxide synthase

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ABSTRACT

A series of N-substituted acetamidines and 2-methylimidazole derivatives structurally related to W1400 were synthesized and evaluated as Nitric Oxide Synthase (NOS) inhibitors. Analogs with sterically hindering isopropyl and phenyl substituents on the benzylic carbon connecting the aromatic core of W1400 to the acetamidine nitrogen, showed good inhibitory potency for nNOS (IC_{50} = 0.2 and 0.3 μM) and selectivity over eNOS (500 and 1166) and to a lesser extent over iNOS (50 and 100). A molecular modeling study allowed to shed light on the effects of the structural modifications on the selectivity of the designed inhibitors toward the different NOS isoforms.

Nitric oxide (NO)[1] is a short-life molecule derived, in biological systems, from L-arginine in a reaction catalyzed by Nitric Oxide Synthases (NOS). Three main isoforms have been identified: two of them are constitutive, the endothelial (eNOS) and the neuronal (nNOS), and one is inducible (iNOS). All three isoforms of NOS catalyze the oxidation of L-arginine to citrulline and NO using reducing equivalents from NADPH in the presence of molecular oxygen.[2]

The constitutively expressed isoform eNOS is involved in the regulation of vascular tone and cell signaling;[3] the other constitutive isoform, nNOS, originally identified in neuronal cells, produces NO that plays important roles in neurotransmitter release and reuptake, neurodevelopment, synaptic plasticity and regulation of gene expression.[4] The expression of iNOS is largely observed in macrophages during inflammation and infection related to host defense;[5] as such, iNOS is a high output enzyme with activity that can be sustained for days leading to cell death and tissue damage.

Since NO is an important signaling molecule in cells, its overproduction may result in pathological effects in virtually every organ system.[6] In particular, in the brain NO is associated with many physiological aspects, including learning and memory, feeding, sleeping and reproductive behavior, as well as in sensory and motor function;[7] however, excessive production of NO following a pathologic insult can lead to neurotoxicity[8] and contributes to neurodegenerative diseases, including stroke,[9] Parkinson's disease,[10] and Alzheimer's disease.[11] Various mechanisms have been identified, including the high levels of iNOS expression in activated glia.[12] Moreover, there is evidence that also NO overproduction by nNOS plays a key role in these pathologies,[13] and recent studies report promising results from chronic inhibition of nNOS as an additional therapeutic strategy for motor nerve repair.[14]

Due to the unique roles played by the three isoforms of NOS in separate tissues, the selective inhibition of an isoform over the others is of paramount importance;[15] in particular, it is essential not to inhibit the eNOS because of its noteworthy role in maintaining blood pressure. The available X-ray structures show a high degree of similarity for the active sites of the three NOS isoforms which makes isoform-selective drug design a challenging task.[16] In the last decade a large amount of studies have been carried out to identify potent and selective inhibitors of iNOS and nNOS as potential treatment of the above pathological conditions.[17,18] Nevertheless, selective inhibitors have been identified, such as *N*-(3-(aminomethyl)-benzyl)acetamidine (**1**, W1400),[19] specific for iNOS, and several N^{ω}-alkyl and N^{ω}-nitro-L-arginine derivatives,[20] specific for nNOS.

The key structural features responsible for the selectivity of the latter inhibitors to nNOS over eNOS and iNOS have been clarified

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by crystallographic and computational studies and rely on subtle differences among the active sites.[21–23] For instance, the selectivity of the N^{ω}-propyl-L-arginine derivatives has been attributed to the different size of the heme ligand binding cavity, which is significantly larger for the nNOS rather than for the eNOS and iNOS and may better accommodate the N^{ω}-propyl group.[22] On the other hand the selectivity of N^{ω}-nitro-L-arginine containing dipeptide inhibitors to nNOS over eNOS recently synthesized by Silverman's group directly depends on a single-residue difference in the periphery of the active site, Asp597 in nNOS and Asn368 in eNOS.[18]

Recently, we have synthesized and evaluated a series of N-substituted acetamidines structurally related to **1** (compounds **2**–**7** in Fig. 1).[24] Among them, compounds characterized by removal of the 3-aminomethyl group of **1** and addition of a methyl or an ethyl group on the benzylic carbon connected to the acetamidine nitrogen, **3** and **4**, respectively, showed good inhibitory potencies toward iNOS and high selectivities over eNOS. A docking study allowed us to shed light on the main effects of the considered structural modifications on interaction with the iNOS isoform, indicating that the interaction of 3-aminomethyl group with the propionate arms of the heme cofactors of iNOS, like **1**, does not contribute significantly to the binding energy and its removal even slightly improves inhibitory potency. On the other hand, the slightly lower selectivity over eNOS indicates a possible role of the 3-aminomethyl group in determining the high isoform specificity of **1**, as suggested by the comparison of the X-ray structures with iNOS and eNOS, which points out the different position of the heme in these two isoforms, leading to different interactions of its propionate arms with the 3-aminomethyl group.

The interesting results obtained prompted us to increase that series of molecules, in order to evaluate the effects of the structural modifications on the inhibitory potency and isoform selectivity of the new compounds and on their interaction within the heme binding site. Here we describe the synthesis, the in vitro evaluation, and a molecular modeling study of new N-substituted acetamidines and 2-methylimidazole derivatives as possible selective nNOS inhibitors (Fig. 2).

Synthesis of compounds *rac*-**9** and **11** was performed according to Scheme 1. From the reaction between 2-methyl-propanenitrile and phenylmagnesium chloride, followed by reduction, 2-methyl-1-phenylpropylamine (**8**) was obtained, while benzhydrylamine (**10**) was obtained by the reduction of diphenylmethanone oxime. Amines **8** and **10** were added, under mild conditions, to *S*-2-naphthylmethyl thioacetimidate hydrobromide, to give desired acetamidines *rac*-**9** and **11**.[25] Condensation of (3-methoxy)benzylamine or (3,4-methylendioxy)benzylamine with ethylacetimidate

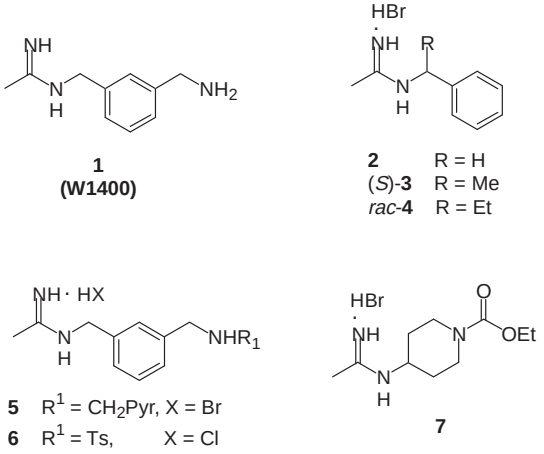


Figure 1. W1400 and selective iNOS inhibitors.

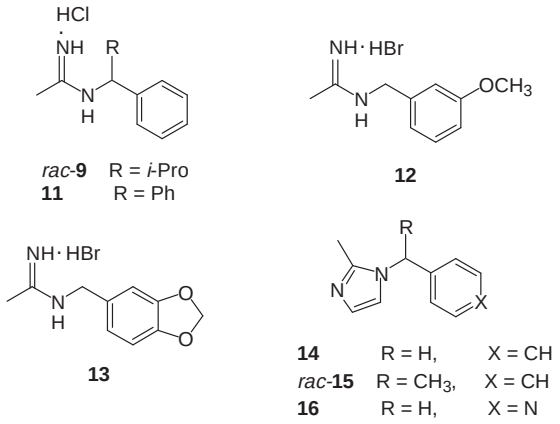
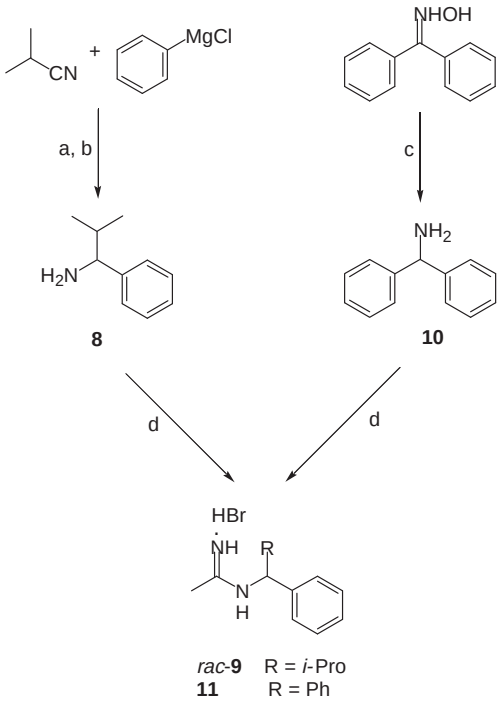
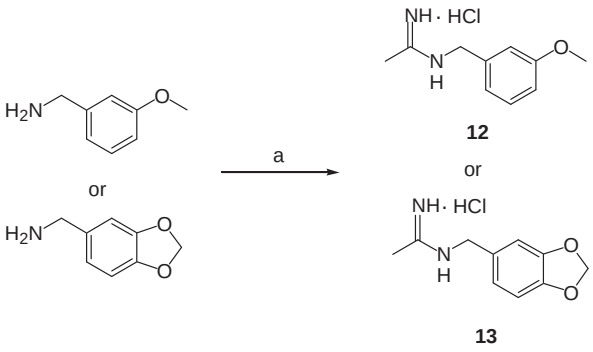


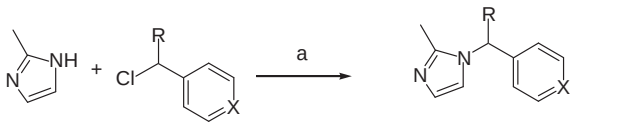
Figure 2. Designed NOS inhibitors.



Scheme 1. Reagents and conditions: (a) dry THF, N_2, reflux, 2 h; (b) $NaBH_4$, dry THF, N_2, reflux, 5 h; (c) Na, abs EtOH, rt, 3 h; (d) *S*-2-naphthylmethyl thioacetimidate hydrobromide, abs EtOH, rt, 2 h.



Scheme 2. Reagents and conditions: (a) ethylacetimidate hydrochloride, NaH, DMF dry, 50 °C, 24 h.



14 R = H, X = CH
rac-**15** R = CH_3, X = CH
16 R = H, X = N

Scheme 3. Reagents and conditions: (a) NaH, dry DMF, 50 °C, 4–48 h.

hydrochloride provided acetamidines **12** and **13**, respectively (Scheme 2).[26] Compounds **14**, *rac*-**15** and **16** were obtained by nucleophilic substitution of chloromethylbenzene, chloroethylbenzene, or chloromethylpyridine, respectively, with 2-methylimidazole (Scheme 3).[27]

All the compounds were biologically evaluated for their capability to inhibit iNOS, eNOS and nNOS, using an enzymatic assay; we used for these isoforms recombinant enzymes from different sources: murine macrophage iNOS, rat brain nNOS, and bovine eNOS. The inhibitory activity against NOS isoforms was measured by following the oxidation of oxyhemoglobin to methemoglobin by NO[28] and the potency results were expressed as IC_{50} values, which are the concentrations that cause 50% loss of activity. The selectivities for iNOS over eNOS (e/i), and for nNOS over the other two isoforms (e/n and i/n) were also evaluated. The known inhibitor **1** was also subjected to the same test system, for comparison; we also included molecules **2**, (*S*)-**3** and *rac*-**4** (Fig. 1) as they were not previously tested on nNOS.

The data presented in Table 1 display for acetamidines **2**, (*S*)-**3** and *rac*-**4**, differing from **1** by the removal of the 3-aminomethyl group and addition of a methyl or an ethyl to the benzylic carbon linked to the amidine group, good iNOS inhibition and poor inhibition of nNOS and eNOS. Our docking studies indicated that the good inhibitory activity of **3** and **4** to iNOS benefits from favorable hydrophobic contacts of the methyl and ethyl groups with the Pro344 and Val346 residues, belonging to the β-sheet S12 which flanks the ligand binding cavity around the heme group. Previous crystallographic studies indicated that the significantly larger size of this cavity for nNOS than for the iNOS and eNOS isoforms is responsible for the selectivity for this isoform of N^{ω}-alkyl-L-arginine inhibitors with substituents of suitable steric hindrance,[22] such as the *n*-propyl group, which fit the nNOS but not the iNOS and eNOS cavities. We thus decided to replace the methyl or ethyl groups of **3** and **4** by more sterically hindering substituents, which could still fit in the cavity of the nNOS but not in those of the iNOS and eNOS isoforms. In agreement with our hypothesis, the increase of the steric hindrance of the substituents on benzylic carbon in **2** led to a dramatic change in the selectivity of these compounds. Indeed, *rac*-**9** and **11**, containing an isopropyl or a phenyl substituent, respectively, displayed a good inhibitory potency against nNOS, and selectivity over iNOS, and especially eNOS. These results show that the inhibitory potency and the selectivity of the above compounds are strongly modulated by the steric hindrance of the substituent on benzylic carbon. While small groups lead to a good inhibition of iNOS and high selectivity over eNOS and nNOS, as in **2**–**4**, more hindered groups lead to a good nNOS inhibition and selectivity over eNOS and to a lesser extent over iNOS, as in **9** and **11**. We then tried to replace the 3-aminomethyl group on the phenyl ring of **1** with other groups containing a different heteroatom such as a methoxy or a dioxolanic system. The introduction of *m*-methoxy group on the phenyl ring of **2**, as in **12**, led to a loss of inhibition toward iNOS with only a moderate increase of inhibition toward nNOS, while that of a dioxolanic system, as in **13**, led to a complete loss of inhibitory potency toward all isoforms. A completely different strategy was employed to design compounds **14**, *rac*-**15**, and **16**, where we combined the features of the above analogs of **1** (without 3-aminomethyl group) with the ligands of heme iron of NOS, bearing an imidazolic ring instead of the acetamidine function. All these 2-methylimidazole derivatives showed a moderate inhibitory potency against nNOS, with limited selectivity over the other isoforms.

Docking simulations were first performed to predict the binding mode in the nNOS, iNOS and eNOS active sites of inhibitors **9** (both as R and S configurations), **11**–**13**, all the analogs of **1** without the 3-aminomethyl group. The effectiveness of the GLIDE program in the docking of **1** on to its natural iNOS substrate was already shown in a previous work to give a good agreement between the calculated pose and the X-ray structure of the **1**-iNOS complex, with a root mean square deviation (RMSD) of 0.341 Å.[24]

The main docked structures of **9** and **11–13** to the three NOS isoforms share the hydrogen bond interactions between the common amidine group and the carboxylate moiety of the Glu371 and Trp366 residues in iNOS or the corresponding Glu592 and Trp587 in nNOS and Glu363 and Trp358 in eNOS. However, quite surprisingly, only the docked poses of **12** and **13** show the same orientation of the benzylic backbone atop the A pyrrole ring of heme while a completely different orientation was observed for **9** and **11**, the compounds with the most sterically hindering isopropyl and phenyl groups and the highest selectivity for nNOS over iNOS and eNOS. In particular, an analysis of the poses obtained for these two compounds show that the isopropyl and phenyl groups apparently do not fit the heme ligand binding cavity as instead observed for the methyl and ethyl groups in our previous docking studies of **3** and **4**. This lack of fit is presumably due to steric clashes of the isopropyl and phenyl groups with the 'back wall'

Table 1
Inhibition of NOS by new acetamidines

Compound	IC_{50} (μM)[a]				Selectivity	
	iNOS	nNOS	eNOS	eNOS/iNOS	eNOS/nNOS	iNOS/nNOS
2	0.2 ± 0.03[b]	150 ± 23	350 ± 18[b]	1750	2.3	0.013
(*S*)-**3**	0.45 ± 0.08b	150 ± 13	300 ± 31[b]	666	2	0.003
rac-**4**	0.25 ± 0.05[b]	90 ± 13	80 ± 8[b]	320	0.9	0.003
rac-**9**	10 ± 1.8	0.2 ± 0.04	100 ± 3	10	500	50
11	30 ± 3	0.3 ± 0.03	350 ± 13	11.6	1166	100
12	50 ± 4	10 ± 1.5	100 ± 9	2	10	5
13	800 ± 26	400 ± 22	400 ± 20	0.5	1	2
14	50 ± 4	5 ± 0.2	90 ± 2	1.8	18	10
rac-**15**	100 ± 2	5 ± 0.6	80 ± 4	0.8	16	20
16	12 ± 2	5 ± 0.3	100 ± 3	8.3	20	2.4
1	0.33 ± 0.06	7.3 ± 1.4	1100 ± 104	3333	150	0.004

[a] The NOS inhibition at 20 μM of arginine. Experiments were independently performed at least three times.
[b] Published data, Ref. 24.

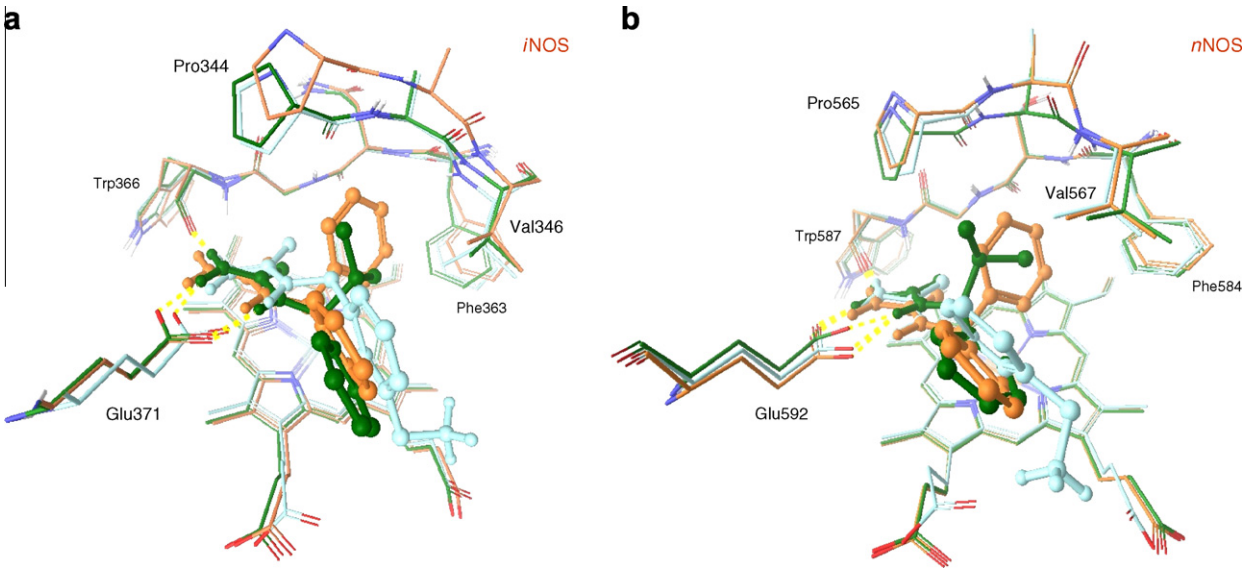


Figure 3. Superposition of (a) nNOS and (b) iNOS bound complexes of **1** (turquoise carbons), **9** (orange carbons), and **11** (green carbons).

made up of the β-sheet S12 (Pro344 to Val346 in the iNOS numbering) which has been shown in our previous docking studies to give hydrophobic contacts with the smaller methyl and ethyl substituents in **3** and **4**. As these favorable hydrophobic contacts were found responsible for the good inhibitory activity of **3** and **4** toward iNOS, we pursued to seek possible poses of **9** and **11** where the isopropyl or phenyl groups fit in this cavity. To this purpose we employed a simple method based on the soft-docking approach[29] to take into account, at least partly, the flexibility of the protein.[30] The soft-docking approach allows to tolerate a significant overlap between ligand and protein atoms during the docking procedure—by reducing their Van der Waals radii—thus permitting the binding of a larger ligand to a pocket structure determined for a complex with a smaller ligand. The highest ranked poses have been selected and submitted to an energy minimization to ensure that any Van der Waals overlaps between protein and ligand atoms can be resolved. Such an approach has been recently validated giving very good results in the foreign-docking of several ligands to moderately flexible proteins.[31] We limited our study to the nNOS and the iNOS which have been shown to possess the largest and the smallest heme binding cavity, respectively. This approach allowed to find several poses of **9** and **11** to nNOS and iNOS in which the isopropyl and phenyl groups fit the heme binding cavity and exploit favorable hydrophobic interactions with the Pro344 and Val346 residues of the β-sheet S12. The most stable of these poses are reported in Figure 3 and compared with the lowest poses for **1** calculated with the same procedure. A significantly higher Molecular Mechanics (MM) binding energy has been calculated for **9** and **11** with the nNOS enzyme than for the iNOS and eNOS isoenzymes in agreement with the selectivity of these two compounds toward nNOS. Moreover, we calculated the reorganization energy required by the two isoforms to accommodate **9** and **11**, and in both cases the penalty for the iNOS isoform is significantly higher (2–3 kcal mol^{-1}) than for the nNOS isoform, as expected on the basis of the smaller heme binding cavity. This higher reorganization energy for the iNOS isoform is probably due to the smaller size of its heme binding cavity and in particular to the shift toward the heme group of the β-sheet S12 in this isoform with respect to nNOS, as shown by the superposition of the X-ray structures of these two isoforms (Fig. 4). These results explain both the isoform selectivity shown by **9** and **11** (due to the highest conformational penalty required by the iNOS isoform to accommodate these ligands) and their good inhibitory activity (due to the favorable contacts of the isopropyl and phenyl groups of these ligands with the hydrophobic residues of the S12 sheet).

In conclusion, in this study we have described a series of *N*-substituted acetamidines and 2-methylimidazole derivatives structurally related to W1400 and screened their activity as selective NOS inhibitors. Analogs *rac*-**9** and **11** showed good inhibitory potency for nNOS (IC_{50} = 0.2 and 0.3 μM, respectively) and selectivity over eNOS (500 and 1166, respectively) and to a lesser extent over iNOS (50 and 100, respectively). A molecular modeling study allowed to elucidate the effects of the structural modifications on isoform activity; in particular, the selectivity of **9** and **11** for nNOS has been attributed to the significantly larger size of the heme binding cavity for nNOS than for the iNOS and eNOS isoforms, which better fit the steric hindering isopropyl and phenyl substituents on the benzylic carbon connecting the aromatic core of **1** to the acetamidine nitrogen.

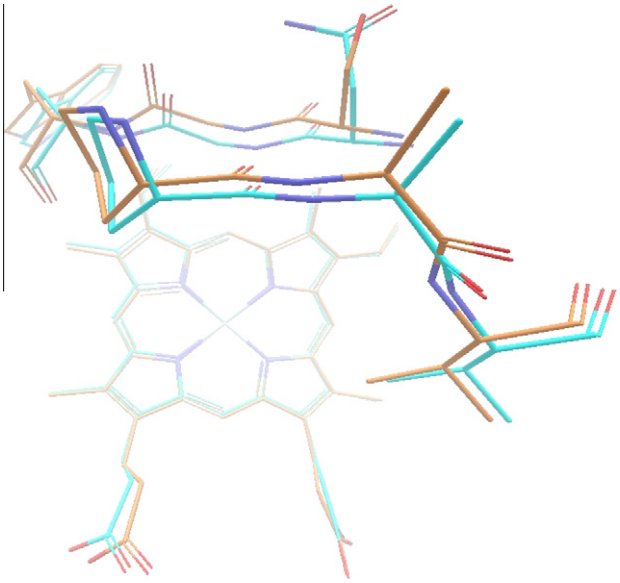


Figure 4. Superposition of the X-ray structures of the iNOS and nNOS isoforms showing the shift of their β-sheet S12.

Acknowledgments

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmcl.2010.09.059.

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