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Original article

Novel inhibitors of nitric oxide synthase with antioxidant properties

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ABSTRACT

We previously described a series of imidazole-based inhibitors substituted at N-1 with an arylethanone chain as interesting inhibitors of neuronal nitric oxide synthase (nNOS), endowed with good selectivity *vs* endothelial nitric oxide synthase (eNOS). As a follow up of these studies, several analogs characterized by the presence of substituted imidazoles or other mono or bicyclic nitrogen-containing heterocycles instead of simple imidazole were synthesized, and their biological evaluation as *in vitro* inhibitors of both nNOS and eNOS is described herein. Most of these compounds showed improved nNOS and eNOS inhibitory activity with respect to reference inhibitors. Selected compounds were also tested to analyze their antioxidant properties. Some of them displayed good capacity to scavenge free radicals and ability to reduce lipid peroxidation.

1. Introduction

The free radical gas nitric oxide (NO) acts as signaling molecule in various tissues and is responsible for both physiological and pathological effects; it is synthesized by nitric oxide synthase (NOS) from L-arginine (L-Arg) and molecular oxygen. Three isoforms of this enzyme have been identified so far: neuronal (nNOS), inducible (iNOS), and endothelial (eNOS) NOS; all three isoforms are constituted by a N-terminal catalytic oxygenase domain which binds Fe-protoporphyrin IX (heme), the substrate L-Arg, and (6R)-5,6,7,8-tetrahydrobiopterin (BH_4), by a central linker region that binds calmodulin (CaM), and by a C-terminal reductase domain with binding sites for flavines (FAD, FMN), and NADPH cofactors; the heme domain provides the site for L-Arg oxidation, while FAD and FMN shuttle electrons from NADPH to the Fe-heme; the cofactor BH_4 is fundamental for the catalytic process, stabilizing the active homodimeric form of the enzyme [1–5].

NO, produced by all three isoforms, is involved in a number of physiological processes in mammalians [6–9]. In particular, NO generated by nNOS is an important neurotransmitter for brain development, memory, learning, brain perception, and long-term potentiation; NO produced by eNOS is responsible for the control of systemic blood pressure and for the inhibition of platelet aggregation; in addition, NO constitutively generated, can serve as neuromodulator in non-adrenergic, non-cholinergic nerve endings of the gastrointestinal, and genitourinary systems; NO derived from iNOS has mainly the biological significance of cytotoxic agent in the normal immune or inflammatory response [10–14].

Nevertheless, overproduction of NO, especially by nNOS and iNOS, is associated with various diseases states such as neurodegeneration, stroke, migraine and chronic headache, Parkinson, Alzheimer, and Huntington diseases (nNOS), hypotensive crises during septic shock, arthritis, colitis, tissue damage, cancer, and various kinds of inflammatory states (iNOS) [15–20]. Besides iNOS overexpression [21–23], recently it has been reported that eNOS is overexpressed in various type of cancer and may be involved in tumor angiogenesis [24–26].

Therefore, the use of substances with inhibitory properties on all the NOS isoforms has great therapeutic potential for the treatment of the above-mentioned diseases. Consequently, over the last twenty years the design and synthesis of NOS inhibitors have received much attention. As a result of both classical and computational studies, some very potent and selective inhibitors of nNOS and iNOS have been nowadays identified [27–32].

At the beginning of our studies on the synthesis of NOS inhibitors, we selected imidazole nucleus as template because imidazole itself and simple imidazole derivatives were reported in the early literature as inhibitors of various isoforms of NOS [33,34]. On these

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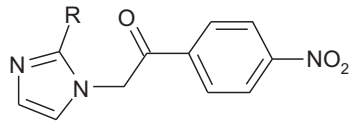
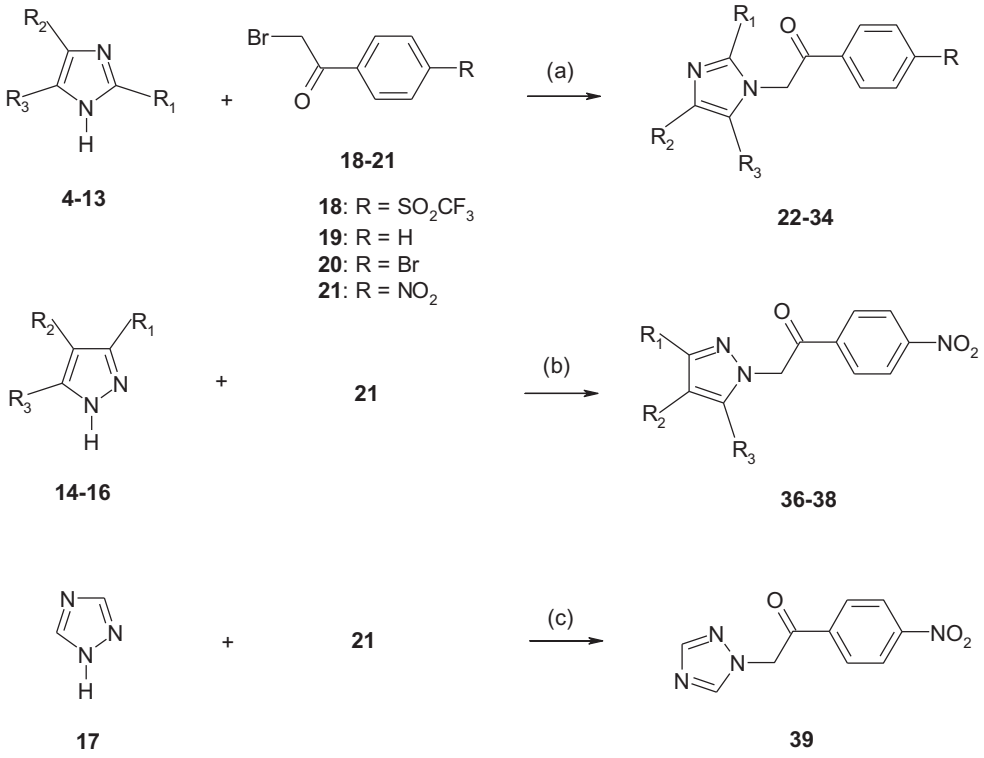


1: R = H
2: R = CH_3
3: R = $CH(CH_3)_2$

Fig. 1. Chemical structures of compounds **1**–**3**.

basis, our research group has conducted extensive investigation of a number of imidazole derivatives variously substituted at the N-1 [35–37]. Some of them showed interesting inhibitory activity on nNOS against eNOS and iNOS, in particular compounds carrying an arylethanone chain; then, we selected the neuronal isoform as main target. Compounds which showed the best biological profile in terms of activity on nNOS and selectivity *vs* eNOS, *i.e.* 2-(1*H*-imidazol-1-yl)-1-(4-nitrophenyl)-ethanone **1** and 2-(2-methyl-1*H*-imidazol-1-yl)-1-(4-nitrophenyl)-ethanone **2** (Fig. 1), were also investigated in order to elucidate their mechanism of action and resulted to inhibit nNOS "non-competitively" *vs* the substrate L-Arg and "competitively" *vs* the cofactor BH_4, without interaction with heme iron [38].



4-13

18-21

18: R = SO_2CF_3
19: R = H
20: R = Br
21: R = NO_2

22-34

14-16

21

36-38

17

21

39

4: R_1 = H, R_2 = H, R_3 = H
5: R_1 = CH_3, R_2 = H, R_3 = H
6: R_1 = H, R_2 = CH_3, R_3 = H
7: R_1 = iC_3H_7, R_2 = H, R_3 = H
8: R_1 = C_3H_7, R_2 = H, R_3 = H
9: R_1 = C_6H_5, R_2 = H, R_3 = H
10: R_1 = H, R_2 = C_6H_5, R_3 = H
11: R_1 = H, R_2 = NO_2, R_3 = H
12: R_1 = C_2H_5, R_2 = CH_3, R_3 = H
13: R_1 = H, R_2 = C_6H_5, R_3 = C_6H_5
14: R_1 = H, R_2 = H, R_3 = H
15: R_1 = CH_3, R_2 = H, R_3 = H
16: R_1 = CH_3, R_2 = H, R_3 = CH_3

22: R = SO_2CF_3, R_1 = H, R_2 = H, R_3 = H
23: R = H, R_1 = CH_3, R_2 = H, R_3 = H
24: R = Br, R_1 = CH_3, R_2 = H, R_3 = H
25: R = NO_2, R_1 = H, R_2 = CH_3, R_3 = H
26: R = NO_2, R_1 = H, R_2 = H, R_3 = CH_3
27: R = H, R_1 = iC_3H_7, R_2 = H, R_3 = H
28: R = Br, R_1 = iC_3H_7, R_2 = H, R_3 = H
29: R = NO_2, R_1 = C_3H_7, R_2 = H, R_3 = H
30: R = NO_2, R_1 = C_6H_5, R_2 = H, R_3 =H
31: R = NO_2, R_1 = H, R_2 = C_6H_5, R_3 = H
32: R = NO_2, R_1 = H, R_2 = NO_2, R_3 = H
33: R = NO_2, R_1 = C_2H_5, R_2 = CH_3, R_3 = H
34: R = NO_2, R_1 = H, R_2 = C_6H_5, R_3 = C_6H_5
36: R_1 = H, R_2 = H, R_3 = H
37: R_1 = H, R_2 = H, R_3 = CH_3
38: R_1 = CH_3, R_2 = H, R_3 = CH_3

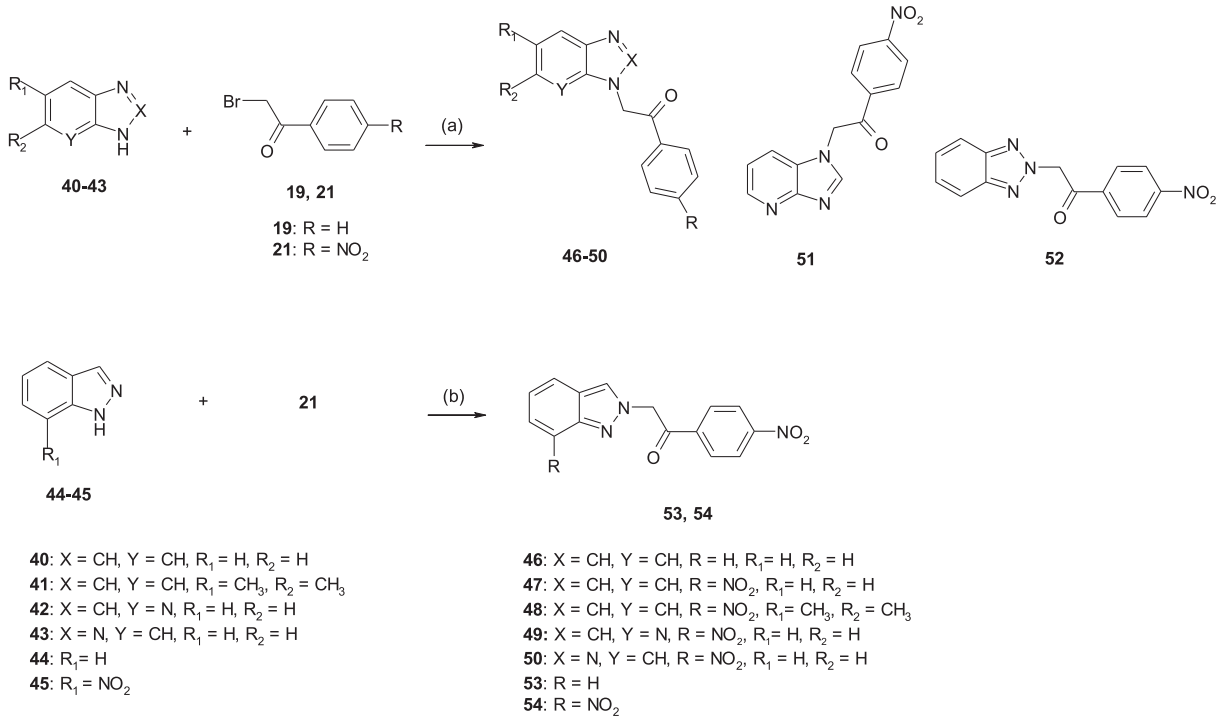
Scheme 1. Reagents and conditions: (a) K_2CO_3, dry DMF, r.t., 1.5 h; (b) $NaNH_2$, dry DMF, 60 °C, 1.5 h; (c) TEA, $CHCl_3$, 0 °C, 4 h.

Some neurological disorders, such as cerebral ischemia and stroke, are related to enhanced formation of free radical species both of nitrogen (NO•) and oxygen ($O_2^{\bullet -}$) in brain tissue [39,40]; in fact inhibition of nNOS as well as scavenging reactive oxygen species has been shown to result in an enhanced neuronal survival after cerebral ischemia [41]. Consequently, combination therapy with substances having different mechanisms of action could be a good strategy for the treatment of the above-mentioned diseases, but often it suffers of the possibility of drug–drug interactions. An alternative approach is to combine multiple activities in the same compound, for example inhibition of nNOS and antioxidant activities [42–44]. Since compounds possessing imidazole ring have been found to exhibit antioxidant activities [45–47], we have recently tested the antioxidant properties of a selected series of imidazole derivatives previously synthesized in our laboratory as nNOS inhibitors, in particular compounds **1**, **2** and 2-[2-(1-methylethyl)-1*H*-imidazol-1-yl]-1-(4-nitrophenyl)-ethanone **3** [48] (Fig. 1). Obtained results showed that compounds **1** and **2** had good capacity to scavenge free radicals and to reduce lipid peroxidation; so, possessing multiple activities in the same molecule, they could be considered useful for therapy of brain diseases caused by an overproduction of radical species, overcoming difficulties derived from a therapy with drugs possessing different mechanisms of action.

With the aim to improve the observed inhibitory activity on nNOS, in this paper we describe the synthesis of a new series of compounds containing an arylethanone chain in which two main structural modifications were carried out with respect to the reference compounds **1**–**3**: (i) Introduction of various substituents at the C-2, C-4, and C-5 of the imidazole nucleus, (ii) Substitution of imidazole nucleus with mono and bicyclic N-containing heterocycles. All synthesized compounds were tested to evaluate the inhibitory properties toward rat recombinant nNOS; selected compounds were also tested on endothelial cell (EC) line eNOS and mouse recombinant iNOS. Some of them were also tested for analyzing their antioxidant properties. To this purpose, their abilities to quench a stable radical, to scavenge superoxide anion, and to inhibit *in vitro* lipid peroxidation, were evaluated.

2. Chemistry

The preparation of title compounds is outlined in Schemes 1 and 2. Synthetic procedures described in literature were taken into account [49–52] and slightly modified. In brief, imidazole derivatives **22**–**34**, benzimidazole derivatives **46**–**48**, pyridoimidazole derivatives **49**, **51**, and benzotriazole derivatives **50** and **52** were obtained by alkylation of heterocycles **4**–**13**, **40**–**43**, respectively, with appropriate 1-aryl-2-bromo-ethanone **18**–**21**, using dry dimethylformamide as solvent, potassium carbonate as base, at room temperature and under nitrogen atmosphere. Pyrazole derivatives **36**–**38** were prepared by alkylation of corresponding pyrazole **14**–**16** with 2-bromo-4′-nitroacetophenone **21** in dry dimethylformamide, sodium amide, at 60 °C, and under nitrogen atmosphere. Triazole derivative **39** was obtained by reaction of 1,2,4 triazole **17** with **21** in chloroform and triethylamine, at 0 °C, under nitrogen atmosphere. Finally, indazole derivatives **53** and **54** were synthesized by reaction of opportune indazole **44** and **45** with an excess of alkylating agent **21** at 120 °C for 1 h in the absence of solvent. Yields were in the range 29–75%. Purification of most compounds was usually performed by flash chromatography. All the synthesized compounds were characterized by analytical, IR and ^{1}H NMR spectral data. The alkylation of some starting azoles potentially can lead to more than one regioisomers; in particular, in some cases one prevalent regioisomer was obtained (**31**, **32**, **33**, **37**, **53**, **54**); in other cases, the formation of a mixture of two regioisomers (**25**, **26**, **49**, **50**, **51**, **52**) occurred; these last isomers were separated by flash chromatography. In order to assign the correct structures for the above-mentioned regioisomers, correlation spectroscopy (nuclear overhauser effect spectroscopy, NOESY) was also used.



Scheme 2. Reagents and conditions: (a) K_2CO_3, dry DMF, r.t., 1.5 h; (b) 120 °C, 1 h.

3. Results and discussion

3.1. NOS inhibition

New synthesized compounds were tested in *in vitro* enzymatic assays to evaluate their ability to inhibit various NOS isoforms. Results, expressed as K_i or percentage of inhibition, are summarized in Table 1, and are in the micromolar range. All compounds were tested on nNOS; compounds were tested on eNOS when K_i nNOS was $\leq$120 μM; compounds that resulted active against nNOS and less active against eNOS were tested also on iNOS. Compounds **1**–**3** (Fig. 1), previously synthesized in our laboratory, were used as reference substances since they have been proven to be good inhibitors of nNOS endowed with good selectivity *vs* eNOS [35]. The first target of this work was to improve this inhibitory activity. Reference compounds **1**–**3** can be considered as constituted by two main portions linked by an ethanone chain, i.e. the imidazole nucleus, and the 4-nitrophenyl moiety. In our previous study we extensively investigated the effect of the substituents on the phenyl ring on nNOS inhibition, concluding that the most potent compounds carried a nitro or a phenyl group at the 4-position [35]. In order to complete this study, in this work firstly we synthesized compound **22** carrying a 4-trifluoromethylsulfonyl substituent, residue able to combine electronic and steric properties of both nitro and phenyl in the same substituent, according to the principal properties approach [53]. Compound **22** resulted less potent than reference compounds, so the nitro group was maintained in the most of the next synthesized compounds. Further modifications on the structure of new potential inhibitors were then realized on the imidazole moiety. Investigation of the mode of action of compounds **1** and **2**, has revealed that these compounds inhibited nNOS "non-competitively" *vs* the substrate L-Arg and "competitively" *vs* the cofactor BH_4, without interaction with heme iron [38]. These results were in contrast with those reported in literature regarding the mechanism of inhibition on various isoforms of NOS caused by simple imidazoles, such as 1, 2, and 4-phenylimidazole [33,34]. These last compounds inhibited nNOS by acting as sixth coordination ligand of the heme iron in the place of oxygen and the order of potency was 1-phenylimidazole > 2-phenylimidazole > 4-phenylimidazole. In terms of structure–activity relationships these results were justified by the reduction of electron density in imidazole nucleus caused by the steric hindrance induced by phenyl ring [33]. On the other hand, we reported that compounds carrying a methyl or 1-methylethyl substituent at the C-2 position of the imidazole nucleus, such as **2** and **3**, were more effective in inhibiting nNOS and less effective in inhibiting eNOS than the unsubstituted analog **1**, affording more selective compounds [35,38]. On these basis and in order to improve the observed inhibitory activity and better understand structure–activity relationships within this class of compounds, in this work we designed and synthesized compounds **23**–**34** (Table 1) characterized by the presence of an imidazole nucleus substituted with methyl, propyl, 1-methylethyl, phenyl or nitro groups at C-2, C-4, and C-5 positions. As a general trend, compounds **23**–**34**, in particular those in which the substituted imidazoles are coupled with a 4-nitrophenylethanone chain, maintained or improved the inhibitory activity on nNOS with respect to reference compounds **1**–**3**. These results confirmed that the presence of 4-nitrophenyl linked to the ethanone chain was critical for nNOS inhibition and that the introduction of a substituent on the imidazole nucleus, with the only exception of 4-NO_2 (**32**), gave a good contribution to the inhibitory properties. Among these substituted imidazoles **23**–**34**, 2-(4-methyl-1*H*-imidazol-1-yl)-1-(4-nitrophenyl)-ethanone **25** was the most interesting since maintained the activity on nNOS and selectivity *vs* eNOS observed for the 2-methyl analog **2**.

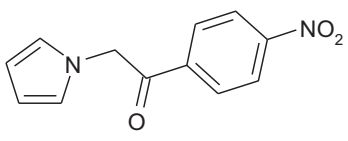
Owing to the close structural class of cytochrome P450 with NOS enzymes, cytochrome P450 inhibition assay was performed for compound **25**. Our results showed that even at concentrations high as 500 μM, compound **25** had no substantial effect (% of inhibition at 500 μM = 13%) on the catalytic activity of microsomal cytochrome P450.

Since results described so far clearly indicated that the presence of unsubstituted imidazole nucleus was not crucial for nNOS inhibition, we designed, synthesized and tested compounds **35**–**39** containing pyrrole, pyrazole or triazole, and compounds **46**–**54** containing benzimidazole, pyridoimidazole, benzotriazole or indazole instead of imidazole. Among monocyclic compounds **35**–**39**, only pyrrole derivative **35** (Table 1), prepared according to reference [54], gave a drastic reduction of activity whereas pyrazole and triazole derivatives **36**–**39** showed inhibitory activity on nNOS comparable or better than reference compounds **1**–**3**, although showing less selectivity *vs* eNOS. Also bicyclic derivatives **46**–**54** were generally more potent toward nNOS than reference compounds **1**–**3**; however, this increase of potency was observed also against eNOS, consequently they resulted less selective than reference compounds. In particular the benzimidazole derivative **47**, being about 17-fold more potent on eNOS than nNOS, could be useful as lead compound to modify in order to improve

Table 1
Inhibitory properties of compounds **22**–**39**, **46**–**54**.

Compound	K_i nNOS (μM)[a]	K_i eNOS (μM)[a]	% of inhibition of iNOS (500 μM)
22	450	NT[b]	NT[b]
23	>500	NT[b]	NT[b]
24	620	NT[b]	NT[b]
25	85	>2000	4
26	90	45	NT[b]
27	750	NT[b]	NT[b]
28	450	NT[b]	NT[b]
29	40	45	NT[b]
30	40	37.5	NT[b]
31	45	70	4.6
32	120	120	NT[b]
33	75	75	NT[b]
34	36.25	25	NT[b]
35[c]	230	NT[b]	NT[b]
36	40	60	30.6
37	63	30	NT[b]
38	65	90	24
39	47.5	40	NT[b]
46	480	NT[b]	NT[b]
47	46.6	2.75	NT[b]
48	13.75	37.5	3.5
49	31.66	70.25	15
50	12.5	32.5	4
51	35.83	45	NT[b]
52	22.5	30	NT[b]
53	32.5	60	12
54	66.25	75	NT[b]
1[d]	105	>5000	31.6
2[e]	74	>2000	32
3[f]	22.5	500	25

[a] Inhibition constants were obtained by measuring percentage of inhibition with at least three concentrations of inhibitor as described in Ref. [66].
[b] Not tested.
[c] Structure of compound **35** [54]:



[d] According to Ref. [35].
[e] According to Ref. [38].
[f] According to Ref. [48].

Table 2
DPPH radical scavenging activities of compounds **25**, **29**, **48**, **50**.

Compound	500 μM[a]	100 μM[a]	50 μM[a]	IC_{50} (μM)
25	41%	17%	12%	>500
29	56%	35%	25%	262
48	45%	19%	11%	>500
50	31%	21%	14%	>500
1[b]				415
2[b]				165
3[b]				230

[a] Values had a standard deviation of ≤10% ($n \geq 3$).
[b] According to Ref. [48].

Table 4
Effect of compounds **25**, **29**, **50** on lipid hydroperoxide levels in human plasma incubated at 37 °C for 2 h.

Compound	100 μM[a]	50 μM[a]	25 μM[a]	IC_{50} (μM)	LogP
25	69%	65%	53%	10	1.48
29	44%	31%	30%	>100	2.17
50	57%	48%	37%	17	2.54
1[b]				18	1.024
2[b]				1.75	1.11
3[b]				>100	1.98

[a] Values had a standard deviation of ≤10% ($n \geq 3$).
[b] According to Ref. [48].

this selectivity [24–26], whereas 5,6-dimethylbenzimidazole derivative **48** and benzotriazole derivative **50** resulted the most interesting compounds in terms of potency against nNOS and selectivity *vs* eNOS.

Results regarding iNOS activity for selected compounds **25**, **31**, **36**, **38**, **48**, **49**, **50**, and **53** evidenced that, even if most of the compounds disclosed showed an increased activity for both nNOS and eNOS isoforms, they were less active toward iNOS isoform with % of inhibition at 500 μM ranging between 4% and 30%.

3.2. Antioxidant properties

The second target of this work was the evaluation of the antioxidant properties of selected compounds endowed with good nNOS inhibition. The aim of combining in the same molecule inhibition of nNOS and antioxidant activities [55] is to reduce neurological damage caused by overproduction of nitrogen and oxygen radical species. Selected compounds were **25** and **29** among monocyclic derivatives, **48** and **50** among bicyclic derivatives. Antioxidant properties were firstly evaluated by two *in vitro* tests: (i) Quenching of 1-diphenyl-2-picrylhydrazyl (DPPH) radical; (ii) Scavenger effect on superoxide anion. Then, compounds **25**, **29**, and **50** were screened in an "*ex vivo cell-free system*" to test lipid peroxidation inhibitory activity. Results are listed in Tables 2–4 and are expressed as percentage of inhibition at different concentrations. IC_{50} values were calculated for the most interesting compounds.

Free radical scavenging activity of the selected compounds was tested by their ability to bleach the stable DPPH radical. This assay provides information on the reactivity of test compounds with a stable free radical. Because of its odd electron, DPPH gives a strong absorption band at $\lambda = 517$ nm in visible spectroscopy (deep violet color). As this electron becomes paired off in the presence of a free radical scavenger, the absorption vanishes, and the resulting decolorization is stoichiometric with respect to the number of electrons taken up. In this assay, most of tested compounds showed a DPPH quenching dose-dependent capacity, usually less than reference compounds **1**–**3** (Table 2). The superoxide anion scavenging capacity of selected compounds was investigated by means of a method which excludes the Fenton-type reaction and the xanthine/xanthine oxidase system. Compounds **25**, **29**, **48**, and **50** inhibited superoxide anion formation in a dose-dependent manner and this activity was generally comparable or better than reference compounds **1**–**3** (Table 3). As a general trend, in this test all compounds resulted more effective than in the previous one. Antioxidant activity in an "*ex vivo cell-free system*" was evaluated by measuring antilipoperoxidative capacity in human plasma incubated with or without some of the synthesized compounds for 2 h at 37 °C. We selected compounds **25**, **29**, and **50** since they were the most active as scavengers of free radicals in the previous assays. All these compounds were able to inhibit lipid peroxidation and their potency was similar or only slightly less than reference compounds **1**–**3** (Table 4). Since lipophilicity can play a role in the capacity of compounds to inhibit lipid peroxidation, usually increasing distribution of the compounds in the membrane lipid bilayer [45], we calculated LogP (cLogP) of compounds **25**, **29**, and **50**. Results show that there is any correlation between cLogP and IC_{50} values because compounds with the highest activity, **25** and **29** (Table 4), showed the lowest and the highest value of cLogP, respectively.

Table 3
Superoxide ion scavenging activities of compounds **25**, **29**, **48**, **50**.

Compound	100 μM[a]	50 μM[a]	25 μM[a]	IC_{50} (μM)
25	56%	23%	20%	66
29	62%	34%	22%	38
48	45%	37%	0%	>100[b]
50	51%	35%	26%	85
1[c]				165
2[c]				70
3[c]				185

[a] Values had a standard deviation of ≤10% ($n \geq 3$).
[b] IC_{50} was not calculated because compound was not soluble at concentration ≥100 μM.
[c] According to Ref. [48].

4. Conclusions

Compounds **22**–**39**, **46**–**59**, containing an arylethanone chain linked to mono or bicyclic nitrogen-containing heterocycles, were synthesized and their inhibitory activity against the three isoforms of NOS was evaluated. Most of them resulted more potent against nNOS and eNOS than reference compounds **1**–**3** and showed very weak activity against iNOS. The most potent inhibitors of nNOS resulted **48** and **50**, whereas the most interesting compound both for potency and selectivity was **25**.

Some compounds (**25**, **29**, **48**, and **50**) were selected to test their ability to quench a stable radical, to scavenge superoxide anion and to inhibit *in vitro* lipid peroxidation. Tested compounds resulted more effective in scavenging superoxide anion than in quenching DPPH radical. Generally, their potency was similar or slightly less with respect to reference compounds **1**–**3**. Compound **25**, being an interesting inhibitor of nNOS endowed with good selectivity *vs* eNOS, iNOS and cytochrome P450, and having good antioxidant properties, emerged from these studies. In consideration of these results, it is reasonable to affirm that the combination of nNOS inhibition and antioxidant activities in the same molecule is possible and is a promising target aimed to provide new therapeutic strategies. Further studies on this class of molecules are in progress to increase selectivity and antioxidant properties.

5. Experimental procedures

5.1. Chemistry: general methods

Melting points were determined in a Gallemkamp apparatus with an MFB-59 digital thermometer in glass capillary tubes and are uncorrected. Elemental analyses for C, H, N were within ±0.4%

of theoretical values and were performed on a Carlo Erba Elemental Analyzer Mod.1108 apparatus. The IR spectra were recorded in KBr disks on a Perkin–Elmer 1600 series FT–IR spectrometer. 1D-^{1}H NMR spectra were determined with a Varian Inova Unity 200 (200 MHz), instrument in DMSO-d_6 solution; ^{13}C NMR spectra and 2D g-COSY and NOESY experiments were determined with a Varian Unity Inova 500 (500 MHz) instrument in $CDCl_3$. Chemical shifts are in δ values (ppm) using tetramethylsilane as the internal standard; coupling constants (J) are given in Hz. Signal multiplicities are characterized as s (singlet), d (doublet), t (triplet), q (quartet), m (multiplet), br (broad signal). All the synthesized compounds were tested for purity on TLC on Merck plates (Kieselgel 60 F_{254}) and spots were visualized under the UV light (λ = 254 and 366 nm). Preparative column chromatography was performed using Merck silica–gel 60 (230–400 mesh). All chemicals and solvents were reagent grade and were purchased from commercial sources. 2-Bromo-1-[(4-trifluromethylsulfonyl)phenyl]-ethanone **18** was prepared according to literature [56]. Analytical and spectral data of compounds **23**, **24**, **46**, and **50**, are in agreement with literature [57–60].

5.2. General procedure for the synthesis of imidazole, benzimidazole, pyridoimidazole, and benzotriazole derivatives ***22–34***, ***46–52***

A solution of imidazole **4–13**, benzimidazole **40**, **41**, pyridoimidazole **42** or benzotriazole **43** (23 mmol) in dry DMF (15 mL) and K_2CO_3 (11.5 mmol) was stirred for 10 min at 0–5 °C, under nitrogen atmosphere. After this time, appropriate 1-aryl-2-bromo-ethanone **18–21** (23 mmol) was added and the mixture was stirred for 1.5 h then poured into ice water. The resulting crude material was extracted with dichloromethane (3 × 50 mL). The combined organic layers were dried over anhydrous sodium sulfate, filtered and concentrated under reduced pressure; the obtained residue were purified by means of flash chromatography performed using silica gel 60 (230–400 mesh) and a mixture of ethyl acetate/methanol 8:2 v/v or dichloromethane/methanol 9.5/0.5 v/v (only for purification of **25** and **26**, **49** and **51**, **50** and **52**, respectively) as eluent. By use of this procedure, the subsequent compounds were obtained:

5.2.1. 2-(1H-Imidazol-1-yl)-1-(4-trifluoromethylsulfonylphenyl)-ethanone (***22***)

The title compound was isolated as yellow powder (38%); mp 98–100 °C; IR (KBr) cm^{-1} 1690, 1373, 1215; ^{1}H NMR (DMSO-d_6) δ 5.84 (s, 2H, CH_2), 6.96 (br s, 1H, imidazole), 7.14 (br s, 1H, imidazole), 7.63 (s, 1H, imidazole), 8.35–8.41 (m, 4H, aromatic). Anal. $C_{12}H_9F_3N_2O_3S$ (C, H, N, S).

5.2.2. 2-(4-Methyl-1H-imidazol-1-yl)-1-(4-nitrophenyl)-ethanone (***25***)

The title compound was isolated as orange powder (45%); mp 144–145 °C; IR (KBr) cm^{-1} 1715, 1527, 1356; ^{1}H NMR (500 MHz, $CDCl_3$) δ 2.29 (s, 3H, CH_3), 5.38 (s, 2H, CH_2), 6.67 (br s, 1H, imidazole), 7.45 (br s, 1H, imidazole), 8.13 (d, J = 8.3 Hz, 2H, aromatic), 8.38 (d, J = 8.3 Hz, 2H, aromatic); ^{13}C NMR (500 MHz, $CDCl_3$) δ 13.3, 52.5, 116.4, 124.4, 129.4, 137.1, 138.6, 138.9, 151.1, 190.6. Anal. $C_{12}H_{11}N_3O_3$ (C, H, N).

5.2.3. 2-(5-Methyl-1H-imidazol-1-yl)-1-(4-nitrophenyl)-ethanone (***26***)

The title compound was isolated as orange powder (29%); mp 179–180 °C; IR (KBr) cm^{-1} 1716, 1524, 1357; ^{1}H NMR (500 MHz, $CDCl_3$) δ 2.13 (s, 3H, CH_3), 5.35 (s, 2H, CH_2), 6.91 (s, 1H, imidazole), 7.46 (s, 1H, imidazole), 8.14 (d, J = 8.4 Hz, 2H, aromatic), 8.39 (d, J = 8.4 Hz, 2H, aromatic); ^{13}C NMR (500 MHz, $CDCl_3$) δ 9.0, 50.9, 124.3, 127.3, 127.6, 129.12, 137.5, 138.5, 151.1, 190.5. Anal. $C_{12}H_{11}N_3O_3$ (C, H, N).

5.2.4. 2-[2-(1-Methylethyl)-1H-imidazol-1-yl]-1-phenyl-ethanone (***27***)

The title compound was isolated as white powder (75%); mp 99–100 °C; IR (KBr) cm^{-1} 2933, 1687; ^{1}H NMR (DMSO-d_6) δ 1.11 (s, 3H, CH_3), 1.52 (s, 3H, CH_3), 2.79–2.94 (m, 1H, CH), 5.70 (s, 2H, CH_2), 6.78 (s, 1H, imidazole), 6.94 (s, 1H, imidazole), 7.56–7.76 (m, 3H, aromatic), 8.05–8.09 (m, 2H, aromatic). Anal. $C_{14}H_{16}N_2O$ (C, H, N).

5.2.5. 1-(4-Bromophenyl)-2-[2-(1-methylethyl)-1H-imidazol-1-yl]-ethanone (***28***)

The title compound was isolated as white powder (70%); mp 154–155 °C; IR (KBr) cm^{-1} 2930, 1694; ^{1}H NMR (DMSO-d_6) δ 1.11 (s, 3H, CH_3), 1.45 (s, 3H, CH_3), 2.79–2.91 (m, 1H, CH), 5.77 (s, 2H, CH_2), 6.79 (d, J = 1.3 Hz, 1H, imidazole), 6.93 (d, J = 1.3 Hz 1H, imidazole), 7.80–7.86 (m, 2H, aromatic), 7.96–8.03 (m, 2H, aromatic). Anal $C_{14}H_{15}BrN_2O$ (C, H, N).

5.2.6. 1-(4-Nitrophenyl)-2-(2-propyl-1H-imidazol-1-yl)-ethanone (***29***)

The title compound was isolated as orange powder (62%); mp 158–160 °C; IR (KBr) cm^{-1} 1693, 1523, 1345; ^{1}H NMR (DMSO-d_6) δ 0.88 (t, J = 7.2 Hz, 3H, CH_3), 1.50–1.71 (m, 2H $CH_2CH_2CH_3$), 2.47 (t, J = 7.2 Hz, 2H, $CH_2CH_2CH_3$), 5.76 (s, 2H, CH_2), 6.78–6.79 (m, 1H, imidazole), 6.98–6.99 (m, 1H, imidazole), 8.26–8.30 (m, 2H, aromatic), 8.40–8.44 (m, 2H, aromatic). Anal.$C_{14}H_{15}N_3O_3$ (C, H, N).

5.2.7. 1-(4-Nitrophenyl)-2-(2-phenyl-1H-imidazol-1-yl)-ethanone (***30***)

The title compound was isolated as orange powder (50%); mp 149–150 °C; IR (KBr) cm^{-1} 1714, 1526, 1354; ^{1}H NMR (DMSO-d_6) δ 5.45 (s, 2H, CH_2), 7.02 (d, J = 1.4 Hz, 1H, imidazole), 7.25 (d, J = 1.4 Hz, 1H, imidazole), 7.38–7.48 (m, 5H, aromatic), 8.02–8.07 (m, 2H, aromatic), 8.30–8.36 (m, 2H, aromatic). Anal. $C_{17}H_{13}N_3O_3$ (C, H, N).

5.2.8. 1-(4-Nitrophenyl)-2-(4-phenyl-1H-imidazol-1-yl)-ethanone (***31***)

The title compound was isolated as orange powder (45%); mp 188–190 °C; IR (KBr) cm^{-1} 1718, 1526, 1354; ^{1}H NMR (500 MHz, $CDCl_3$) δ 5.47 (s, 2H, CH_2), 7.25–7.28 (m, 2H, aromatic + imidazole), 7.39 (dd, J = 7.2 and 7.8 Hz, 2H, aromatic), 7.59 (s, 1H, imidazole), 7.83 (d, J = 7.8 Hz, 2H, aromatic), 8.17 (d, J = 8.4 Hz, 2H, aromatic), 8.41 (d, J = 8.4 Hz, 2H, aromatic). ^{13}C NMR (500 MHz, $CDCl_3$) δ 52.7, 115.7, 124.3, 124.8, 127.1, 128.6, 129.2, 133.8, 138.1, 138.5, 142.9, 151.1, 190.4. Anal. $C_{17}H_{13}N_3O_3$ (C, H, N).

5.2.9. 1-(4-Nitrophenyl)-2-(4-nitro-1H-imidazol-1-yl)-ethanone (***32***)

The title compound was isolated as orange powder (56%); mp 223–224 °C; IR (KBr) cm^{-1} 1682, 1584, 1482, 1256, 1092; ^{1}H NMR (500 MHz, $CDCl_3$) δ 5.55 (s, 2H, CH_2), 7.50 (s, 1H, imidazole), 7.84 (s, 1H, imidazole), 8.19 (d, J = 8.6 Hz, 2H, aromatic), 8.45 (d, J = 8.6 Hz, 2H, aromatic). ^{13}C NMR (500 MHz, $CDCl_3$) δ 54.8, 123.2, 124.4, 129.9, 138.8, 139.1, 147.2, 150.9, 192.1. Anal. $C_{11}H_8N_4O_5$ (C, H, N).

5.2.10. 2-(2-Ethyl-4-methyl-1H-imidazol-1-yl)-1-(4-nitrophenyl)-ethanone (***33***)

The title compound was isolated as orange powder (65%); mp 138–139 °C; IR (KBr) cm^{-1} 1702, 1529, 1345; ^{1}H NMR (500 MHz, $CDCl_3$) δ 1.32 (t, J = 7.6 Hz, 3H, CH_2CH_3), 2.24 (s, 3H, CH_3), 2.56 (q, J = 7.6 Hz, 2H, CH_2CH_3), 5.28 (s, 2H, CH_2), 6.53 (s, 1H, imidazole),

8.14 (d, $J = 8.7$ Hz, 2H, aromatic), 8.40 (d, $J = 8.7$ Hz, 2H, aromatic). ^{13}C NMR (500 MHz, $CDCl_3$) δ 12.2, 13.6, 20.0, 51.9, 116.5, 124.4, 129.1, 136.7, 138.9, 148.9, 151.1, 191.4. Anal.$C_{14}H_{15}N_3O_3$ (C, H, N).

*5.2.11. 2-(4,5-Diphenyl-1H-imidazol-1-yl)-1-(4-nitrophenyl)-ethanone (**34**)*

The title compound was isolated as orange powder (49%); mp 193–195 °C; IR (KBr) cm^{-1} 1714, 1603, 1526, 1354; ^{1}H NMR (DMSO-d_6) δ 5.62 (s, 2H, CH_2), 7.10–7.29 (m, 5H, aromatic), 7.38–7.41 (m, 5H, aromatic), 7.83 (s, 1H, imidazole), 8.08–8.19 (m, 2H, aromatic), 8.24–8.40 (m, 2H, aromatic). Anal. $C_{23}H_{17}N_3O_3$ (C, H, N).

*5.2.12. 2-(1H-Benzimidazol-1-yl)-1-(4-nitrophenyl)-ethanone (**47**)*

The title compound was isolated as orange powder (65%); mp 189–190 °C; IR (KBr) cm^{-1} 1709, 1518, 1348; ^{1}H NMR (DMSO-d_6) δ 6.12 (s, 2H, CH_2), 7.17–7.27 (m, 2H, aromatic), 7.55–7.60 (m, 1H, aromatic), 7.66–7.71 (m, 1H, aromatic), 8.18 (s, 1H, aromatic), 8.31–8.36 (m, 2H, aromatic), 8.43–8.47 (m, 2H, aromatic). Anal. $C_{15}H_{11}N_3O_3$ (C, H, N).

*5.2.13. 2-(4,5-Dimethyl-1H-benzimidazol-1-yl)-1-(4-nitrophenyl)-ethanone (**48**)*

The title compound was isolated as orange powder (68%); mp 203–205 °C; IR (KBr) cm^{-1} 2931, 1710, 1520, 1351; ^{1}H NMR (DMSO-d_6) δ 2.28 (s, 3H, CH_3), 2.31 (s, 3H, CH_3), 6.04 (s, 2H, CH_2), 7.33 (br s, 1H, aromatic), 7.44 (br s 1H, aromatic), 8.01 (s, 1H, aromatic), 8.30–8.38 (m, 2H, aromatic), 8.42–8.48 (m, 2H, aromatic). Anal. $C_{17}H_{15}N_3O_3$ (C, H, N).

*5.2.14. 2-(3H-Imidazo[4,5-b]pyridin-3-yl)-1-(4-nitrophenyl)-ethanone (**49**)*

The title compound was isolated as orange powder (34%); mp 197–199 °C; IR (KBr) cm^{-1} 1702, 1522, 1350; ^{1}H NMR (500 MHz, $CDCl_3$) δ 5.83 (s, 2H, CH_2), 7.31 (dd, $J = 4.0$ and 7.0 Hz, 1H, aromatic), 8.15 (d, $J = 7.0$ Hz, 1H, aromatic), 8.18 (s, 1H, aromatic), 8.28 (d, $J = 8.8$ Hz, 2H, aromatic), 8.39 (d, $J = 4.0$ Hz, 1H, aromatic), 8.42 (d, $J = 8.8$ Hz, 2H, aromatic). ^{13}C NMR (500 MHz, $CDCl_3$) δ 48.9, 118.8, 124.3, 128.4, 129.3, 134.9, 138.6, 144.1, 144.5, 146.8, 151.0, 190.5. Anal. $C_{14}H_{10}N_4O_3$ (C, H, N).

*5.2.15. 2-(1H-Imidazo[4,5-b]pyridin-1-yl)-1-(4-nitrophenyl)-ethanone (**51**)*

The title compound was isolated as orange powder (48%); mp 166–168 °C; IR (KBr) cm^{-1} 1708, 1519, 1349; ^{1}H NMR (500 MHz, $CDCl_3$) δ 5.68 (s, 2H, CH_2), 7.30 (dd, $J = 5.1$ and 6.9 Hz, 1H, aromatic), 7.61 (d, $J = 6.9$ Hz, 1H, aromatic), 8.19 (s, 1H, aromatic), 8.24 (d, $J = 8.2$ Hz, 2H, aromatic), 8.45 (d, $J = 8.2$ Hz, 2H, aromatic), 8.65 (d, $J = 5.1$ Hz, 1H, aromatic). ^{13}C NMR (500 MHz, $CDCl_3$) δ 51.2, 118.7, 124.7, 128.1, 129.2, 135.9, 138.7, 144.2, 145.2, 147.2, 151.0, 189.7. Anal. $C_{14}H_{10}N_4O_3$ (C, H, N).

*5.2.16. 2-(2H-Benzotriazol-2-yl)-1-(4-nitrophenyl)-ethanone (**52**)*

The title compound was isolated as orange powder (72%); mp 166–168 °C; IR (KBr) cm^{-1} 1698, 1519, 1349; ^{1}H NMR (500 MHz, $CDCl_3$) δ 6.21 (s, 2H, CH_2), 7.44 (dd, $J = 2.8$ and 6.7 Hz, 2H, aromatic), 7.90 (dd, $J = 2.8$ and 6.7 Hz, 2H, aromatic), 8.17 (d, $J = 8.9$ Hz, 2H, aromatic), 8.37 (d, $J = 8.9$ Hz, 2H, aromatic). ^{13}C NMR (500 MHz, $CDCl_3$) δ 62.1, 118.2, 124.1, 127.2, 129.3, 138.7, 145.1, 151.2, 189.2. Anal. $C_{14}H_{10}N_4O_3$ (C, H, N).

*5.3. General procedure for the synthesis of pyrazole derivatives **36**–**38***

A solution of appropriate pyrazole **14**–**16** (23 mmol) in dry DMF (15 mL) and $NaNH_2$ (23 mmol) was stirred for 10 min at 60 °C, under nitrogen atmosphere. After this time, 2-bromo-4′-nitroacetophenone **21** (23 mmol) was added and the mixture was stirred for 1.5 h. The reaction mixture was chilled and poured into ice water and the resulting crude material was extracted with dichloromethane (3 × 50 mL). The combined organic layers were dried over anhydrous sodium sulfate, filtered and concentrated under reduced pressure; the obtained residue was purified by means of flash chromatography performed using silica gel 60 (230–400 mesh) and a mixture of ethyl acetate/methanol 8:2 v/v as eluent. By use of this procedure, the subsequent compounds were obtained:

*5.3.1. 1-(4-Nitrophenyl)-2-(1H-pyrazol-1-yl)-ethanone (**36**)*

The title compound was isolated as orange powder (57%); mp 206–207 °C; IR (KBr) cm^{-1} 1710, 1517, 1348; ^{1}H NMR (DMSO-d_6) δ 5.92 (s, 2H, CH_2), 6.29–6.35 (m, 1H, pyrazole), 7.49 (d, $J = 1.4$ Hz, 1H, pyrazole), 7.75 (d, $J = 1.8$ Hz, 1H, pyrazole), 8.23–8.27 (m, 2H, aromatic), 8.37–8.41 (m, 2H, aromatic). Anal. $C_{11}H_9N_3O_3$ (C, H, N).

*5.3.2. 2-(4-Methyl-1H-pyrazol-1-yl)-1-(4-nitrophenyl)-ethanone (**37**)*

The title compound was isolated as orange powder (43%); mp 115–117 °C; IR (KBr) cm^{-1} 2983, 1709, 1519, 1351; ^{1}H NMR (500 MHz, $CDCl_3$) δ 2.31 (s, 3H, CH_3), 5.54 (s, 2H, CH_2), 6.17 (s, 1H, pyrazole), 7.40 (s, 1H, pyrazole), 8.14 (d, $J = 8.2$ Hz, 2H, aromatic), 8.35 (d, $J = 8.2$ Hz, 2H, aromatic). ^{13}C NMR (500 MHz, $CDCl_3$) δ 13.3, 57.7, 106.5, 123.8, 124.1, 129.3, 131.6, 139.0, 151.2, 191.6. Anal. $C_{12}H_{11}N_3O_3$ (C, H, N).

*5.3.3. 2-(3,5-Dimethyl-1H-pyrazol-1-yl)-1-(4-nitrophenyl)-ethanone (**38**)*

The title compound was isolated as orange powder (65%); mp 122–124 °C; IR (KBr) cm^{-1} 2993, 1710, 1517, 1348; ^{1}H NMR (DMSO-d_6) δ 2.08 (s, 3H, CH_3), 2.11 (s, 3H, CH_3), 5.76 (s, 2H, CH_2), 5.87 (s, 1H, pyrazole), 8.21–8.27 (m, 2H, aromatic), 8.35–8.42 (m, 2H, aromatic). Anal. $C_{13}H_{13}N_3O_3$ (C, H, N).

*5.4. Preparation of 1-(4-nitrophenyl)-2-(1H-1,2,4-triazol-1-yl)-ethanone (**39**) [51]*

A solution of triazole **17** (10 mmol) in $CHCl_3$ (50 mL) and TEA (10 mmol) was stirred for 10 min at 0–5 °C. After this time, 2-bromo-4′-nitroacetophenone **21** (10 mmol) was added and the mixture was stirred for 4 h, maintaining the temperature between 0 and 5 °C. The solvent was removed under vacuum, the oil residue was poured into ice water, and the resulting crude material was extracted with dichloromethane (3 × 50 mL). The combined organic layers were dried over anhydrous sodium sulfate, filtered and concentrated under reduced pressure; purification by means of flash chromatography performed using silica gel 60 (230–400 mesh) and ethyl acetate as eluent gave the title compound (62%) as orange powder; mp 162–164 °C; IR (KBr) cm^{-1} 1694, 1519, 1346; ^{1}H NMR (DMSO-d_6) δ 6.09 (s, 2H, CH_2), 8.05 (s, 1H, triazole), 8.22–8.38 (m, 2H, aromatic), 8.40–8.45 (m, 2H, aromatic), 8.52 (s, 1H, triazole). Anal. $C_{10}H_8N_4O_3$ (C, H, N).

*5.5. General procedure for the synthesis of indazole derivatives **53** and **54***

A mixture of the appropriate indazole **44** and **45** (10 mmol) and 2-bromo-4′-nitroacetophenone **21** (20 mmol) was heated in an oil bath for 1 h at 120–130 °C. After cooling, the reaction mixture was treated with 15 mL of ethyl acetate and the precipitate obtained was collected and dried. The solid was suspended in water, treated with stoichiometric amount of 1 N NaOH, and stirred for 1 h. The resulting

mixture was extracted with dichloromethane (3 × 50 mL), the combined organic layers were dried over anhydrous sodium sulfate, filtered and concentrated under reduced pressure. Recrystallization of the residue from ethanol gave the desired products as solids.

*5.5.1. 2-(2H-Indazol-2-yl)-1-(4-nitrophenyl)-ethanone (**53**)*

The title compound was isolated as orange powder (73%); mp 198–200 °C; IR (KBr) cm^{-1} 1706, 1520, 1348; ^1H NMR (500 MHz, $CDCl_3$) δ 5.91 (s, 2H, CH_2), 7.12 (dd, $J = 8.3$ and 7.1 Hz, 1H, aromatic), 7.33 (dd, $J = 8.3$ and 7.2 Hz, 1H, aromatic), 7.70 (dd, $J = 8.3$ and 9.6 Hz, 2H, aromatic), 8.06 (s, 1H, aromatic), 8.20 (d, $J = 8.8$ Hz, 2H, aromatic), 8.37 (d, $J = 8.8$ Hz, 2H, aromatic). ^{13}C NMR (500 MHz, $CDCl_3$) δ 59.3, 117.5, 120.2, 122.4, 124.2, 124.4, 125.5, 126.8, 129.5, 138.8, 149.4, 150.8, 190.4. Anal. $C_{15}H_{11}N_3O_3$ (C, H, N).

*5.5.2. 2-(2H-7-Nitroindazol-2-yl)-1-(4-nitrophenyl)-ethanone (**54**)*

The title compound was isolated as orange powder (66%); mp 214–216 °C; IR (KBr) cm^{-1} 1712, 1520, 1531, 1348, 1333; ^1H NMR (500 MHz, $CDCl_3$) δ 6.13 (s, 2H, CH_2), 7.29 (dd, $J = 8.2$ and 7.7 Hz, 1H, aromatic), 8.14 (d, $J = 8.2$ Hz, 1H, aromatic), 8.23 (d, $J = 8.4$ Hz, 2H, aromatic), 8.37 (s, 1H, aromatic), 8.38–8.44 (m, 3H, aromatic). ^{13}C NMR (500 MHz, $CDCl_3$) δ 60.0, 120.7, 124.5, 125.7, 127.5, 128.7, 129.7, 136.2, 137.9, 138.3, 140.9, 143.2, 189.4. Anal. $C_{15}H_{10}N_4O_5$ (C, H, N).

5.6. Enzyme assays

5.6.1. NOS isoenzymes

Neuronal rat recombinant nitric oxide synthase isolated from a Baculovirus overexpression system in SF9 cells, was purchased from ALEXIS. Inducible recombinant nitric oxide synthase isolated from mouse macrophages were purchased from Sigma–Aldrich. Endothelial nitric oxide synthase was prepared by a transformed endothelial cell (EC) line from the heart of C57BL/6 mice (H5V). EC (passage 5) were cultured in flasks (Falcon, Becton Dickinson) until confluent in Dulbecco's modified Eagle's medium (Life Technologies), with 10% fetal calf serum, 1 mM glutamine and antibiotics and incubated at 37 °C in a humidified 5% CO_2 atmosphere. The medium was changed every 2 days and subcultures were performed every 4–5 days following treatment with trypsin-EDTA. ECs were scraped and washed in phosphate-buffered saline. Approximately 1×10^9 ECs were suspended in 1.5 mL of 50 mM Tris–HCl pH 7.4 containing 10 mM EDTA, 5 mM glucose, 1.15% w/v KCl, 0.1 mM DTT, 2 mg/l leupeptin, 2 mg/l pepstatin, and 44 mg/l phenylmethylsulfonyl fluoride. The cell suspensions were homogenized by sonication twice for 5 s with a Soniprep and then centrifuged at 0 °C (20,000× *g* for 20 min). The supernatant was then used for the enzymatic assay.

5.6.2. Enzymatic assay

The assay for NO synthase activities was carried out according to Hevel and Marletta [61], measuring the rate of conversion of oxyhemoglobin to methemoglobin using a Hitachi UV 2000 spectrophotometer. A reference cuvette was charged with 5 mM oxyhemoglobin (human A_0, purchased from SIGMA) in 100 mM Hepes (pH 7.4) to a final volume of 500 μl. A typical sample contained: 50 μM L-arginine, imM Mg^{++} (required only for iNOS), 170 μM Dithiothreitol (DTT), 100 μM NADPH, 4 μM FAD, 12 μM BH_4, 1 mM Ca^{++} (required only for eNOS and nNOS), 20 U/ml Calmodulin (required only for eNOS and nNOS), 10 μl of DMSO (or the same volume of DMSO solution of test compounds to a final concentration of 500, 100 and 50 mM), enzymatic extract (1.2 U/ml for nNOS and iNOS and supernatant derived for 1×10^9 ECs eNOS), and 5 μM oxyhemoglobin in 100 mM Hepes, pH 7.4. The Hepes buffer was preheated prior to use. Under these conditions, the NO formed reacts with oxyhemoglobin to yield methemoglobin which can be measured at $\lambda = 401$ nm.

5.6.3. Cytochrome P450 activity assay

Rat spleen microsomal *Cyt P450* activity was measured by following the NADPH-dependent reduction of cytochrome *c* as described in our previous research [62]. Incubations were done at 25 °C for 15 min in absence and presence of different concentrations of compound **25** (5–500 μM). Reaction rates were determined by reading the absorbance of reduced cytochrome *c* at 550 nm.

5.7. Antioxidant tests

5.7.1. Chemicals

Nicotinamide-adenine dinucleotide, reduced form disodium salt, 1-diphenyl-2-picrylhydrazyl radical (DPPH), xylenol orange were obtained from Sigma–Aldrich Co. (St. Louis, MO, USA). All other chemicals were from Merck (Frankfurt, Germany).

5.7.2. Methods

All the imidazole derivatives analyzed show a major absorption in the 220–260 nm range.

5.7.2.1. Scavenger effect on superoxide anion. Superoxide anion was generated *in vitro* as described by Russo et al. [63]. A total volume of 1 mL of the assay mixture contained: 100 mM triethanolamine-diethanolamine buffer, pH 7.4, 3 mM NADH, 25 mM/12.5 mM EDTA/$MnCl_2$, 10 mM β-mercapto-ethanol; samples contained different concentrations of compounds **25**, **29**, **48**, and **50** (25–50–100 μM). After 20 min incubation at 25 °C, the decrease in absorbance at $\lambda = 340$ nm was measured. Results are expressed as percentage of inhibition of NADH oxidation with respect to control. SOD (40 mU/ml) was used as reference compound.

5.7.2.2. Quenching of DPPH. The free radical-scavenging capacity of different concentrations of **25**, **29**, **48**, and **50**, was tested by their ability to bleach the stable 1,1-diphenyl-2-picrylhydrazyl radical (DPPH) [64]. The reaction mixture contained 86 μM DPPH and different concentrations of compounds **25**, **29**, **32**, **48**, and **50** (50–100–500 μM) in 1 mL of ethanol. After 10 min at room temperature the absorbance was recorded at 517 nm. Results are expressed as percentage decrease in absorbance at $\lambda = 517$ nm with respect to control. Trolox (20 μM) was used as reference compound.

5.7.2.3. Determination of lipid hydroperoxide levels in the plasma of a healthy donor. Plasmatic lipid hydroperoxide levels were evaluated by oxidation of Fe^{+2} to Fe^{+3} in the presence of xylenol orange (FOX assay) at $\lambda = 560$ nm [65]. Heparinized venous blood was collected after overnight fasting; plasma was separated by centrifugation at 800 g for 20 min. Plasma aliquots (500 μl) were diluted 1:1 with oxygenated PBS and incubated at 37 °C for 2 h with or without different concentrations of compounds **25**, **29**, and **50** (10–50–100 μM) in a total volume of 1 mL. Calibration was obtained using hydrogen peroxide (0.2–20 μM). Results are expressed as percentage of inhibition respect to control (plasma incubated in absence of test compounds).

5.7.3. Partition coefficient

Calculation of cLogP of compounds **25**, **29**, and **50**, was afforded using an Advanced Chemistry Development (ACD/Labs) Software Solaris (4.67).

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